Oncogenic signalling in t(12;21) Acute Lymphoblastic Leukaemia

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DECLARATION

I hereby declare that this thesis is my own. Where other sources of information have been used, I confirm that these have been indicated in the thesis.

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ABSTRACT

The t(12;21)(p13;q22) translocation is present in up to 25% of children with pre-B cell Acute Lymphoblastic Leukaemia (ALL). This translocation involves two transcription factors, TEL (ETV6) and AML (RUNX1), both of which have crucial roles in regulating haematopoiesis. Clinically, TEL-AML1 positive patients have good prognoses. However, late relapses, additional genetic lesions affecting prognosis, and long-term side-effects of chemotherapy remain a cause for concern. In light of recent studies showing genetic and functional heterogeneities in cells responsible for cancer clone maintenance and propagation, targeting common deregulated pathways may be critical for the success of novel therapies.

Using Affymetrix GeneChip global gene expression analysis our laboratory previously identified three genes: *Tbx2, E2f5* and *Lif-R,* specifically expressed in TEL-AML1 transduced mouse foetal liver haematopoietic progenitor cells (HPC) cells compared with control cells. Over-expression of these genes was confirmed by real-time qPCR and the specificity of target gene expression was evaluated in human TEL-AML1 positive and negative leukaemia cells. Pathway analysis of TEL-AML1 transcriptional target genes also demonstrated deregulated expression of genes associated with STAT3 signalling, known to be one of the most important pathways required for proliferation and maintenance of multipotency in cancer stem cells.

In this study we demonstrate the importance of STAT3 activity in a mouse model of TEL-AML1 overexpression, in human TEL-AML1 positive leukaemia cells and primary human leukaemic samples. Our data indicate a central role for TEL-AML1 in maintaining activated STAT3. This is mediated by transcriptional induction of the Guanine nucleotide exchange factor, ARHGEF4, leading to RAC1 activation and consequent stimulation of STAT3. The latter is necessary for survival, proliferation and self-renewal of TEL-AML1 positive leukaemia through transcriptional induction of MYC expression. In conclusion, we show a novel signalling pathway important for maintenance of t(12;21) leukaemia that constitutes a promising novel therapeutic target for the treatment of this disease.

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ABBREVIATIONS

AGM	Intraembryonic aorta-gonad-mesonephros
2-ME	2-mercaptoethanol
ABL	Abelson murine leukaemia viral oncogene homolog 1
AKT	Thymoma viral oncogene homolog
ALL	Acute Lymphoblastic Leukaemia
AML	Acute Myeloid Leukaemia
AML1	Acute myeloid leukaemia 1 protein
APC	Allophycocyanin
APC	Adenomatous polyposis coli
APRF	Acute phase response factor
BCL-2	B-cell lymphoma 2
BCR	Breakpoint cluster region
BM	Bone marrow
BMI1	Polycomb ring finger oncogene
CBF	Core binding factor
CFU	Colony-forming unit
ChIP	Chromatin immunoprecipitation
CLPs	Common lymphoid progenitors

CML	Chronic myeloid leukaemia
CMPs	Common myeloid progenitor
c-MYC	v-myc avian myelocytomatosis viral oncogene homolog
CNTF	Ciliary neurotrophic factor
CSF	Colony stimulating factor
CT	Cardiotrophin-1
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DH	Dbl-homology
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulphoxide
E2F5	E2F transcription factor 5
EBF-1	Early B-cell factor 1
EDTA	Ethylenediaminetetraacetic acid
EdU	5-ethynyl-2´-deoxyuridine
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ENU	*N*-ethyl-*N*-nitrosourea
ETS	E-twenty six

ETV6	ETS translocation variant 6
FERM	Four-point-one, ezrin, radixin, moesin
GAPs	GTPase-Activating Proteins
GDIs	Guanosine nucleotide dissociation inhibitors
GEFs	Guanine nucleotide exchange factors
GIST	Gastro-intestinal stromal tumour
GLI1	Growth factor independence 1
GLUT	Glucose transport proteins
GMPs	Granulocyte/monocyte progenitors
HAT	Histone acetyltransferase
HCSs	Haematopoietic stem cells
HDACs	Histone deacetylases
hESCs	Human embryonic stem cells
HGF	Hepatocyte growth factor
HLH	Helix-loop-helix
Hoxa-9	Homeobox A9
HPCs	Haematopoietic progenitor cells
Hpgk	Human phosphoglycerate kinase eukaryotic promoter
HTLV	Human T-lymphotropic virus

IAP	Inhibitor of apoptosis
IFNs	Interferons
IKZF1	IKAROS
IL-7R	IL-7 receptor
INT	2-(P-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride
JAK	Janus kinase
LDL	Low density lipoprotein
LIF	Leukaemia inhibitory factor
LIF-R	Leukaemia inhibitory factor receptor
LMPPs	Lymphoid-primed multipotent progenitors
MAPK	Mitogen-activated protein kinase
MCL1	Myeloid cell leukaemia sequence 1
MEPs	Megakaryocytic/erythroid progenitors
MFI	Mean fluorescence intensity
MLL	Mixed-lineage leukaemia
MPLs	Lymphoid progenitor cells
MPPs	Multipotent progenitors
NK	Natural killer
NLS	Nuclear localization signal

NSCLC	Non-small cell lung cancer
OSM	Oncostatin M
PAX5	Paired box 5
PBS	Phosphate buffered saline
PcGs	Polycomb group proteins
PD	Pointed domain
PDGFR	Platelet-derived growth factor receptor
PE	Phycoerythrin
PH	Pleckstrin homology
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-Kinase
pRb	Retinoblastoma protein
PVDF	Polyvinylidenefluoride
qRT-PCR	Quantitative real-time polymerase chain reaction
RAS	Rat sarcoma
RHD	Runt Homology Domain
RIPA	Radio-Immunoprecipitation Assay
RPMI	Roswell Park Memorial Institute
RUNX	Runt-related transcription factor

SB	Sleeping Beauty
shRNA	Small hairpin ribonucleic acid
SLAM1/CD150	Signalling lymphocyte activation molecule family member 1
SPIB	Spi-B transcription factor
STAT3	Signal transducer and activator of transcription 3
TA	Transcriptional activating domain
TBS	Tris-buffered saline
TBX2	T-box transcription factor 2
TCA	Tricarboxylic acid
TEL	Translocation–Ets-leukemia
TGFβ	Transforming growth factor beta
TYK2	Tyrosine kinase 2
v-SRC	Sarcoma viral oncogene homolog tyrosine kinase
WNT	Wingless-type

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CHAPTER I

1.1 Haematopoiesis

Haematopoiesis is the name given to the process of blood cell production. Millions of blood cells are replaced by new ones every second during our life, making blood the most regenerative and plastic tissue present in the human body (Rieger and Schroeder, 2012). This characteristic is effective during emergency situations, such us infection or during bleeding, where blood cell counts have to rapidly increase. All the different blood cell types arise from a small population of cells called haematopoietic stem cells (HSCs) that during adult life reside mainly in the bone marrow (BM), the major site of adult haematopoiesis (Rieger and Schroeder, 2012).

Most of the studies to understand haematopoiesis have been done in mice and currently this is the best understood of all species. The use of genetically modified mice for the gain- or loss- of function of specific genes controlling haematopoiesis has facilitated experimental analysis of haematopoiesis *in vivo*. Moreover, the use of conditional mutants, that confer specific expression of particular genes only in haematopoietic cells, has further permitted the study of the function of many genes in different cell lineages (de Boer et al., 2003; Kuhn et al., 1995). However, haematopoiesis has also been studied in other experimental organisms. The use of animal models such as the *Zebrafish* and *Drosophila melanogaster* is revealing many important insights,

mainly due to the faster and cheaper genetic manipulation of these organisms (Crozatier and Vincent, 2011; Jing and Zon, 2011).

In human, as in mice, the generation of the first cells with the functional property to regenerate the haematopoietic system upon transplantation into recipient mice, that is the principal characteristic of HSCs, occurs in the intraembryonic aorta-gonad-mesonephros (AGM) region and the placenta (Gekas et al., 2005; Ivanovs et al., 2011). Before this, haematopoietic cells are generated firstly in the extraembryonic yolk sac then in the allantois and placenta. In addition, there is also evidence for production of transient populations of blood cells in the early stages of the embryo. Thus, primitive erythrocytes, that still have the nucleus, primitive macrophages and megakaryocytes are present in the extraembryonic yolk salk before HSCs and then disappear as the embryo develops further (Chen et al., 2011). After being generated in the AGM, HSCs migrate to the foetal liver, the major site for their expansion. Shortly thereafter they migrate to the spleen and around birth to the bone marrow, which becomes the main site of HSC-derived haematopoiesis after birth (Figure 1-1). The tissue origin of these HSCs has only recently been solved in mice. Thanks to time-lapse microscopy, Eilken and colleagues were able to detect a continuous endothelial to haematopoietic transition in mice, subsequently confirmed by another two studies in *Zebrafish* embryos (Bertrand et al., 2010; Eilken et al., 2009; Kissa and Herbomel, 2010). The spatial and temporal similarities that exist between mouse and human haematopoiesis

suggest that the same mechanisms may also be involved in human embryonic haematopoiesis.

During adult haematopoiesis, all blood lineages derive from a small number of HSCs mainly located in the bone marrow. These cells have two different capacities, they have the potential to self-renew and they can differentiate into various haematopoietic progenitor cells (HPCs), which in turn are further able to differentiate in all the blood lineages (Till and McCulloch, 1980). The generation of new cells occurs in a hierarchical manner, HSCs being located at the apex and cells lower down in the hierarchy having an increasingly restricted lineage potential. The different populations of cells are mainly identified by multiple surface markers (lineage markers). Although there are many similarities between human and mouse haematopoiesis, HSCs from the two species express different markers. Human HSCs, in contrast to mouse HSCs, in fact do not express the markers Sca1 and the signalling lymphocyte activation molecule family member 1 (SLAM1/CD150) (Larochelle et al., 2011). Additionally, while the $CD34^{+}/CD38^{-}$ phenotype was regarded as the canonical marker set for human HSCs, mouse HSCs are mainly located within the $CD34^{-}/CD38^{+}$ population (Matsuoka et al., 2001; Tajima et al., 2001). The highest purity of human HSCs can be found within the $Lin^{-}/CD34^{+}/CD34RA^{-}/CD90^{+}/Rhodamine123^{low}/CD49f^{+}$ population (Notta et al., 2011). However, a very recent paper has challenged this distinction, showing not only that a population of $CD34^{-}$ cells with HSC features is present in human bone marrow, but that it can also be placed above the $CD34^{+}$ population in the

haematopoietic hierarchy suggesting more similarity between mouse and human haematopoiesis than previously appreciated (Anjos-Afonso et al., 2013).

From this small population, cells differentiate into a cascade of progenitors with diminishing multilineage potential, before unilineage commitment occurs. The first population that arises from HSCs is the multipotent progenitor (MPP) subset that retains multi-lineage differentiation potential but differs from the original population in lacking self-renewal ability (Reya, 2003). The existence of this population has been discovered both in human and mouse. From these cells arise two different populations of progenitors. The first one, that loses the ability to give rise to erythroid and megakaryocytic lineages, is the early lymphoid progenitor cell population [MLPs in human and lymphoid-primed multipotent progenitor (LMPPs) in mouse] that is largely lymphoid restricted but possesses also myeloid, but not erythroid and megakaryocytic, potential. MLPs/LMPPs generate B and T lymphocytes and natural killer (NK) cells, but they can also differentiate into granulocyte/monocyte progenitors (GMPs) or completely differentiated myeloid cells (Adolfsson et al., 2005). The second population is the common myeloid progenitor population (CMP), that produces GMPs and megakaryocyte/erythroid progenitors (MEPs) which differentiate into monocytes and granulocytes, and erythrocyte and megakaryocytes, respectively (Doulatov et al., 2012) (Figure 1-2).

1.1.1 Molecular control of haematopoiesis

Transcription factors play fundamental roles during haematopoiesis, controlling stem cell maintenance, lineage commitment and differentiation. In HSCs, transcriptional regulation is focused on maintaining the capacity for self-renewal and promotion of cell survival. There are many factors involved in the control of self-renewal. One of the most important genes able to maintain self-renewal is Homeobox A9 (*Hoxa-9*). This gene is the highest expressed of the Hox family in HSC and knock-out studies demonstrated that HSC derived from Hoxa-$9^{-/-}$ mice were unable to reconstitute the haematopoietic system of transplanted recipient mice (Lawrence et al., 2005). The same result is also found following ablation of the growth factor independence 1 (*Gli1*) gene, presumably due to the fact that the encoded factor is a repressor of p53 activity, a factor essential for the quiescent status of HSCs (Liu et al., 2009; Zeng et al., 2004). Another group of genes important for HSC self-renewal encode the polycomb group proteins (PcGs). In particular, animals deficient for the polycomb ring finger oncogene (*Bmi1*), a member of this family, showed reduced levels of HSC self-renewal activity and an increased rate of differentiation (Iwama et al., 2004). The balance between stem cell self-renewal and differentiation has been shown also to be controlled by the v-myc avian myelocytomatosis viral oncogene homolog (*c-MYC*) presumably by controlling the interactions between HSCs and their niche by regulating the expression of N-cadherin and integrins (Wilson et al., 2004). The main factors responsible for HSC survival are the proteins belonging to the B-cell lymphoma 2 (BCL-2)

family, such as the myeloid cell leukaemia sequence 1 (MCL1) factor (Opferman et al., 2005; Orelio and Dzierzak, 2007). All these functions are regulated by extracellular stimuli that control pathways regulating networks of transcription factors. The main pathways responsible for the regulation of self-renewal in HSCs are the wingless-type MMTV integration site family (WNT) and the Janus kinase (JAK)- signal transducer and activator of transcription (STAT) pathways. The WNT ligands bind to a receptor complex consisting of a member of the Frizzled family and the low density lipoprotein (LDL) receptor-related proteins LRP5 or LRP6. In the canonical WNT pathway, receptor activation leads to stabilization of β-catenin, which accumulates and translocates to the nucleus where it activates target gene expression (Clevers, 2006). Addition of WNT proteins or over-expression of the constitutive active form of β-catenin leads to increased self-renewal activity *in vitro* and haematopoietic reconstitution ability *in vivo* (Reya and Clevers, 2005). Many cytokine receptors that lack catalytic activity can transduce signals into the cell through the JAK-STAT pathway (see section 5.1). Due to the wide variety of stimuli that can activate this signalling pathway it can profoundly influence HSCs self-renewal. For example, activation of STAT5, one of the main mediators of the pathway, was shown to induce long-term self-renewal in human HSCs (Fatrai et al., 2011; Schuringa et al., 2004).

Extracellular stimuli are also capable of influencing haematopoietic lineage choice. Cytokines, extracellular matrix and membrane-bound signalling molecules can influence and regulate lineage commitment. These stimuli are

converted inside the cells into gene expression changes by networks of transcription factors that regulate the cell fate choice. Cytokines, can be lineage specific or can regulate cells in multiple lineages, and, for some cell types, the concurrent action of different cytokines is required for the final outcome. The main group of cytokines responsible mainly for myeloid lineage commitment is the colony stimulating factor (CSF) family. There are four different CSFs, each with differing colony-stimulating activity. The given working names indicate the most numerous type of colony stimulated in colony forming assays: GM-CSF (CSF2) stimulates granulocyte and macrophage colony formation; M-CSF (CSF1) stimulates macrophage colony formation; G-CSF (CSF3) stimulates granulocyte colony formation; and multi-CSF (IL-3) stimulates a broad range of haematopoietic cell colony types (Metcalf, 2010). One of the main cytokines responsible for lymphoid commitment is IL-7. Mice deficient for IL-7 or any component of its receptor exhibit severely impaired B and T lymphopoiesis since the common lymphoid progenitor (CLP) compartment is dramatically reduced in these mutant mice (Peschon et al., 1994; Puel et al., 1998; Tsapogas et al., 2011; von Freeden-Jeffry et al., 1995). Evidence that cytokines can direct the lineage commitment comes also from experiments demonstrating the ability of cytokines to redirect specification in cells already committed to a particular lineage. Ectopic expression of the IL-2 receptor in CLPs, for instance, can convert their cell fate from lymphoid to myeloid (Kondo et al., 2000). Similarly, GM-CSF receptor signalling can redirect IL-7 receptor-

deficient lymphoid progenitors to the myeloid cell lineage (Iwasaki-Arai et al., 2003)

These experiments support an inductive model for haematopoietic differentiation (Metcalf, 1991). In contrast to this model, an alternative hypothesis has been proposed stating that intrinsic molecular events within cells determine their lineage commitment, known as stochastic haematopoietic development (Ogawa, 1994). Supporting the latter model, different lineage-specific transcription factors have been shown to be co-expressed in multipotent cells before lineage commitment and these proteins often inhibit each other transcriptionally or form multiprotein complexes that are not able to function normally (Palani and Sarkar, 2009). For this reason, random fluctuations of transcription factor expression could lead to a particular one gaining dominance and thereby regulating the differentiation process. In the stochastic model, moreover, cytokine signalling has been seen as a secondary event to transcription factor lineage specification, in the sense that transcription factor activated cytokine receptor expression permits the survival and amplification of the stochastically committed cells (Cross et al., 1997) (Figure 1-3).

1.1.2 B-cell biology and development

B-lymphocyte cells develop in the bone marrow from HSCs. The B-cell specification process is best understood in the mouse where lymphoid

commitment starts at the lymphoid-primed multipotent progenitor (LMPPs) stage and continues in common lymphoid progenitors in order to initiate B cell specification through the pre-pro B stage and the pro-B stages where the rearrangement of the immunoglobulin heavy chain (IgH) locus is initiated. Successful IgH gene rearrangement generates pre-B cells that express the pre-B-cell receptor (pre-BCR) and these then undergo rearrangement of the Ig light chain loci to further differentiate into immature B cells. This commitment requires a hierarchical regulation of many transcription factors that has been mostly elucidated. Generally, during these transitions, gene expression associated with multipotency and stemness gives way to expression of genes associated with the B cell fate (Figure 1-4).

One of the first key regulators is IKAROS (IKZF1), a transcription factor with an N-terminal conserved zinc-finger domain that mediates DNA binding and a C-terminal zinc-finger domain required for homo- and heterodimerization. IKZF1 can function either as a transcriptional activator or repressor, recruiting various different chromatin remodelling complexes (Kim et al., 1999). It plays a fundamental role in lymphoid commitment and specification since *Ikzf1*$^{-/-}$ mice are completely deficient in NK cells, B and T cells and, specifically, in B cell development they lack the pre-pro-B cell transitional population (Georgopoulos et al., 1994; Wang et al., 1996). This data was further confirmed with homozygous mice bearing a hypomorphic *Ikzf1* allele (Kirstetter et al., 2002). The main function of this transcription factor in lymphoid development is to suppress the expression of genes associated with stemness and myeloid

associated genes and to induce expression of lymphoid specific genes (Ng et al., 2009). Another essential regulator at the LMPP stage of lymphoid specification and restriction of myeloid potential is E2A, a protein belonging to the E protein (class I) family of helix-loop-helix transcription factors (Dias et al., 2008). Progenitor cells lacking of this factor fail to generate pro-B cells (Zhuang et al., 1994). Moreover, similar to IKZF1, E2A is able to regulate the expression of important factors required for B-cell commitment. These two transcription factors cooperate to induce B cell lineage specific genes, including two of the main genes necessary for B cell commitment. Thus, they are both able to regulate the expression of the gene encoding for the IL-7 receptor (IL-7R) (Dias et al., 2008; Yoshida et al., 2006). This expression is necessary for complete B specification since deficiency in the IL-7R, or IL-7 itself, leads to an early block in B cell development, specifically at the pro-B stage (Peschon et al., 1994; von Freeden-Jeffry et al., 1995). This block is mainly due to impaired recombination of the VH gene segment that is regulated mainly by the STAT proteins (Corcoran et al., 1998; Goetz et al., 2004). Furthermore, they also regulate the expression of the early B-cell factor 1 (*EBF1*) gene. Interestingly, the factor encoded by this gene is able to rescue the aberrant B lymphoid phenotype in Ikz1$^{-/-}$ mice and *E2a* deficient cells (Reynaud et al., 2008; Seet et al., 2004; Smith et al., 2002). The importance of this gene has been also shown in *Ebf1*$^{-/-}$ mice, where its absence results in an absolute block in differentiation at the pre-pro-B stage (Lin and Grosschedl, 1995). The key function of EBF1 is to induce the expression of the guardian and main regulator of B-cell identity,

Paired box 5 (PAX5). *Pax5* gene expression is initiated at the pro-B cell stage (Fuxa and Busslinger, 2007) and during early stages of commitment is subject to allele-specific regulation with only one allele being transcribed in any given cell. This switches to bi-allelic expression only at later stages of B cell commitment (Nutt et al., 1999b). Similar to the other B cell specific transcription factors, PAX5 is known to maintain B cell identity through the transcriptional activation of B cell specific genes and repression of genes associated with other lineages. Interestingly, the set of target genes regulated by PAX5 is different in early and late B lymphopoiesis, suggesting an essential role for this factor for the final commitment (Revilla et al., 2012). Experiments with *Pax5* deficient embryos showed, moreover, a lack of B lymphopoiesis in the foetal liver whereas B cell development proceeded to a progenitor-cell stage in the bone marrow suggesting a differential requirement for *Pax5* in foetal and adult B lymphopoieisis (Nutt et al., 1997; Urbanek et al., 1994). However, the bone marrow progenitor cells were not committed to the B lineage, as *in vitro* culture of these cells in presence of specific lineage cytokines allowed them to differentiate into functional macrophages, granulocytes, dendritic cells, osteoclasts and NK cells (Nutt et al., 1999a). Additionally, conditional inactivation of *Pax5* in mature peripheral B cells resulted in de-differentiation to lymphoid progenitors which could give rise to functional T cells *in vivo* (Cobaleda et al., 2007a).

As already mentioned activation of B cell-specific genes and simultaneously shutting down the expression of B cell inappropriate genes is

the fundamental function of PAX5 during B cell lineage commitment. Regulation of the expression of genes involved in the signal transduction of the pre-BCR and, subsequently of the BCR, such as CD19 and CD21 indicates that PAX5 plays a fundamental role for the pre-B to differentiate into a population of cells, named immature B cells, ready to leave the bone marrow to further differentiate in the peripheral lymphoid organs (Cobaleda et al., 2007b). Thus, during this stage the cells are subjected to negative and positive selection in order to select for B cells with functional surface expression of BCR and eliminate B cells with overt reactivity to self-antigen, ensuring maintenance of immunological self-tolerance (Sandel and Monroe, 1999). After this selection, cells migrate into peripheral tissues and remain inactive until they encounter pathogen. Depending of the antigen source, full stimulation of B cells can be (thymus-dependent activation) or not (thymus-independent activation) mediated by T cells. The more common activation is given by activated antigen-specific helper T cells, which stimulate B cells to proliferate and form germinal centres where they differentiate into memory B cells or plasma cells responsible of antibody secretion.

1.1.3 Alterations in haematopoietic transcription factor genes

Genetic alterations, such as mutations, deletions or translocation, of many of the genes encoding transcription factors essential for foetal haematopoiesis and lineage commitment, in most cases cause severe haematopoietic problems or even embryonic death. Appropriate expression of

genes during haematopoietic development and differentiation is extremely important and the failure of these mechanisms may lead to arrested differentiation and cellular transformation, ultimately causing the disease known as leukaemia.

1.2 Acute lymphoblastic leukaemia

The haematopoietic progenitor cells of each committed lineage are also called blasts, for example lymphoblasts or myeloblasts, and in normal conditions they represent less than 5% of the cells made in the bone marrow. When the blasts of one lineage represent more than 20% of the total amount of cells and, in some cases, are found in the peripheral blood, this is considered a pathological status known as leukaemia (Harris et al., 1999). Leukaemia is clinically and pathologically subdivided into a variety of large groups. The first division is between its acute and chronic forms. Subsequently, it is subdivided according to which kind of blood progenitor cell is affected. This split divides leukaemia into lymphoblastic and myeloid leukaemia (Harris et al., 1999).

Acute Lymphoblastic Leukaemia (ALL) is a malignant disorder of lymphoid progenitor cells. It affects both children and adults, but the peak prevalence is between the ages of 2 and 5 years, and it is the most commonly occurring cancer in children, representing 30% of all paediatric tumours. Although the overall survival rate is currently almost 90%, it remains one of the main causes of cancer deaths in children (Pui et al., 2011). Moreover, in

developed countries the incidence of this disease has increased by approximately 1% per year over the past two decades (Linabery and Ross, 2008).

The general mechanisms underlying the induction of ALL include the aberrant expression of proto-oncogenes, chromosomal translocations that create fusion genes encoding active kinases or altered transcription factors, and hyperdiploidy (Chen et al., 2010). These genetic alterations contribute to the leukaemic transformation of hematopoietic stem cells or their committed progenitors by changing cellular functions. In fact, they can alter key regulatory processes by maintaining or enhancing an unlimited capacity for self-renewal, subverting the controls of normal proliferation, blocking differentiation, and promoting resistance to death signals (apoptosis) (Pui et al., 2004). The majority of ALLs are represented by a pre-B-cell phenotype, displaying cell surface marker expression associated with normal pre-B cells, although approximately 20% also exhibit aberrant myeloid lineage specific cell surface antigen expression (Firat et al., 2001). However, the leukaemic cells are blocked at this particular stage of the differentiation process and accumulate in the body (Inaba et al., 2013).

1.2.1 t(12;21) Acute Lymphoblastic Leukaemia

One of the main alterations associated with ALL is the balanced chromosomal translocation that often involves the in-frame fusion of genes

encoding transcription factors which play critical roles in normal haematopoiesis. The most frequent single gene recombination in paediatric cancer is the t(12;21)(p13;q22) rearrangement, representing more than the 20% of all paediatric acute lymphoblastic leukaemia cases (Mullighan, 2012) (Figure 1-5). This translocation involves two genes encoding transcription factors: ETS translocation variant 6 (*ETV6*) also known as translocation–Ets-leukemia (*TEL)* that maps on chromosome 12 and Runt-related transcription factor 1 (*RUNX1*) also known as Acute myeloid leukemia 1 protein (*AML1*) that maps on chromosome 21 (Romana et al., 1994).

Both TEL (ETV6) and AML1 (RUNX1) are transcription factors involved in normal haematopoiesis. They have been shown to be essential for the establishment of haematopoiesis of all lineages in the bone marrow and constitutive loss of either one of the genes encoding them is embryonic lethal (Wang et al., 1998). *AML1* belongs to the Runt-related transcription factor (*RUNX*) family of genes, also called core binding factor (*CBF*) genes, and its expression is primarily restricted to cells of the haematopoietic lineage (Corsetti and Calabi, 1997). It is required for hematopoiesis in the embryonic stage but, in contrast, loss of *AML1* in the adult does not cause complete loss of hematopoiesis. Specifically, it has been shown, using conditional knockout mice, that *Aml-1* is required for maturation of megakaryocytes and differentiation of T and B cells, but not for maintenance of HSC during adult haematopoiesis (Ichikawa et al., 2004). Moreover, a recent paper showed that it is necessary for survival and development of B-cell-specified progenitors

showing, in addition, the occupancy of enhancer regions of genes critical for pre-B-cell transition (Niebuhr et al., 2013). The highly evolutionarily conserved Runt domain is responsible for both DNA binding and heterodimerization with the partner protein, core-binding factor β (CBFβ) (Figure 1-6A). Although the protein alone is able to regulate the expression of many genes, binding to the CBFβ subunit increases DNA binding affinity and protects RUNX1 from ubiquitin-proteosome-mediated degradation (Huang et al., 2001). The *TEL* gene encodes the E-twenty six **(**ETS) family transcription factor ETV6, which is an essential regulator of post-natal HSCs. It is required for the establishment of hematopoiesis of all lineages in the bone marrow. Hematopoietic stem cells or progenitors need this factor in order to migrate or home to the bone marrow from the foetal liver, or to respond appropriately to the signal from the bone marrow niche and survive within the bone marrow microenvironment (Hock et al., 2004; Wang et al., 1997; Wang et al., 1998). The protein has three main domains: the N-terminal pointed domain (PD) which mediates protein-protein interactions, including ETV6 oligomerisation, a C-terminal DNA-binding domain and a central domain that is mainly involved in the recruitment of repression complexes (Poirel et al., 1997) (Figure 1-6B).

The translocation between these two genes creates a fusion transcript that includes sequences encoding the PD domain of TEL, but not its DNA-binding domain, and almost the entire coding region of *AML1* (Figure 1-6C). Although the presence of the t(12;21) translocation is detectable years before the clinical onset of disease, and occurs most likely *in utero*, on its own it is not

sufficient to induce frank leukaemia (Zelent et al., 2004) (Figure 1-7). Indeed it generates a pre-leukaemic clone that requires secondary mutations in order to induce leukaemic transformation. One of the more recent hypotheses suggests that acute lymphoblastic leukaemia could be a result of an abnormal response to a common infection. Based on this idea, two different possible mechanisms have been suggested. According to Kinlen's "population mixing" theory, ALL could arise as a consequence of non-immune children exposed to some unknown infection(s), through population movement and mixing (Kinlen, 1995). In contrast, a distinct hypothesis, known as "delayed infection theory", states that ALL may arise from a lack of exposure to infectious agents at a younger age. Later, this deficiency may result in an abnormal immune response to otherwise common infections, which would create a favorable environment to promote leukemic transformation (Greaves, 2006).

There is a large discrepancy between the frequency of normal cord blood samples positive for the presence of the translocation and the incidence of ALL. Some studies suggest that only 1 of 100 newborns with detectable *TEL-AML1* transcripts are destined to develop ALL (Mori et al., 2002). In contrast, others propose a model in which the initiating event is as rare as the disease itself, implying that a high proportion (perhaps 100%) of babies born with the TEL-AML1 fusion are destined to develop leukaemia (Lausten-Thomsen et al., 2011). This difference in frequency is still a matter for debate but the "two hit" model best explains the biology of the disease, suggesting that the secondary genetic alteration is necessary and may represent the limiting

factor for the development of the disease (Mori et al., 2002) (Figure 1-8). This is also suggested by analysis of blood from healthy adults that also demonstrated the presence of the fusion gene (Olsen et al., 2006).

In patients, this translocation is detected in leukaemic blasts that appear to be blocked at an early stage of B-lymphoid lineage differentiation, when they initiate but fail to complete immunoglobulin gene rearrangement (Panzer-Grumayer et al., 2005; Pui et al., 2004; Sloma and Eaves, 2009). The most immature TEL-AML1^{+} population is in fact identified by the aberrant CD34^{+}CD38^{-}CD19^{+} phenotype, that has been associated with a very early stage of lineage commitment (Hong et al., 2008). Thus far, no experimental models of t(12;21) leukaemia in mice have been able to recapitulate a definite B-ALL phenotype, regardless of the timing and mode of TEL-AML1 expression. The first generation of *TEL-AML1* transgenic mice was performed using the immunoglobulin heavy chain enhancer/promoter to drive the expression of the human fusion gene in lymphoid cells. But although expression was confirmed, the mice did not develop a malignant haematological disorder or any signs of alteration in the haematopoiesis (Andreasson et al., 2001). In addition, a different approach was used by several groups, transplanting into irradiated mice bone marrow cells transduced with a retrovirus expressing the fusion gene. In one of the studies, over-expression of the fusion caused leukaemia in two of the nine transplanted mice (one T-lineage ALL and one B-precursor ALL) whereas in the other two only accumulation of both multipotent and B-cell progenitors was shown (Bernardin et al., 2002; Fischer et al., 2005; Tsuzuki et

al., 2004). A similar study, but using foetal haematopoietic cells, exhibited, instead, an increase in repopulation of myeloid cells and B cells, although the differentiation of TEL-AML1$^+$ cells remained unperturbed (Morrow et al., 2004). Two more recent studies created knock-in mouse models where *TEL-AML1* expression was driven from the endogenous *Tel* promoter (Schindler et al., 2009; van der Weyden et al., 2011). Expression of the fusion gene alone did not cause disease in either study, but increased numbers of HSC and a transient increase in foetal B progenitor self-renewal was observed in one of the models (Schindler et al., 2009). However, both mouse models exhibited predisposition to development of leukaemia following mutagenesis. In particular, Schindler and colleagues showed that induction of T-cell leukaemia following treatment with the *N*-ethyl-*N*-nitrosourea (ENU) mutagen was faster and more penetrant in knock-in mice compared to controls. More interestingly, van der Weyden and colleagues, using the *Sleeping Beauty* (SB) transposase, were able to obtain B-ALL in almost 20% of the mice (Schindler et al., 2009; van der Weyden et al., 2011).

The difficulty in accurately modelling TEL-AML1$^+$ ALL may be due to weak oncogenic activity of this fusion, which may require secondary genetic changes to complete the transformation from the pre-leukaemic to the leukaemic clone. This possibility is supported by studies on ALL in identical twins. Although both twins frequently contain cells with the identical *TEL-AML1* fusion, the latency of ALL is often markedly different in each affected individual, with one of the twins in some cases failing to develop the disease at all (Ford et

al., 1998; Hong et al., 2008). The knock-in mouse model generated by Schindler et al demonstrated that TEL-AML1 expression in HSC results in a persistent pre-malignant population susceptible to the accumulation of further genetic hits for the progression to malignancy (Schindler et al., 2009). The most frequent secondary genetic abnormalities include the loss of the DNA region encompassing the second allele of *TEL* and deletion of part of chromosome 9, including the *PAX5* and *CDKN2A* loci (Mullighan et al., 2007). However, the multistep process that ultimately results in overt leukaemia remains poorly understood.

The biology of pre-leukaemic TEL-AML1^{+} cells is not well understood either. Some evidence suggests that pre-leukemic clones can be propagated after B-lineage commitment. In fact, analysis of immunoglobulin receptor rearrangements and retroviral transduction of *TEL-AML1* in human and mouse models suggested that the fusion may promote self-renewal of early B cells (Ford et al., 1998; Hong et al., 2008; Morrow et al., 2004). Recent findings, moreover, demonstrate that TEL-AML1 may play a role in down-regulating the expression of genes encoding the Spi-B transcription factor (SPIB) and IKZF3, two regulators of the pre-B transition, suggesting a function of the fusion in blocking the B cell specification process (Niebuhr et al., 2013). Taken together, these findings could explain the block in B cell differentiation and the accumulation in the blood of pre-leukemic clones (Niebuhr et al., 2013).

The most common hypothesis for how TEL-AML1 functions is that it acts as an antagonist of endogenous RUNX1 transcriptional activity. Transient over-

expression of the fusion protein is able to repress the activity of reporter constructs of haematopoietic-specific genes and/or antagonize RUNX1 dependent transcriptional activity (Fears et al., 1997; Hiebert et al., 1996; Uchida et al., 1999). This repression may be due to the ability of ETV6 to recruit nuclear receptor/histone deacetylase (HDACs) complexes, mainly through the PD, such as mSin3A, n-CoR and HDACs, forming stable repressor complexes (Petrie et al., 2003; Wang and Hiebert, 2001) (Figure 1-9). However, recent evidence from Panzer-Grümayer's group also suggests that TEL-AML1 may play a role in promoting the expression of particular downstream target genes. Although the conclusion of this work was in agreement with the hypothesis that TEL-AML1 represses RUNX1 target genes, the macro-array analysis, following shRNA-mediated knock-down of the fusion gene in human leukaemic cell lines, revealed that PI3K/AKT/mTOR signalling and the HSC gene expression signatures were highly enriched in the list of down-regulated genes (Fuka et al., 2011). In particular the PI3K/AKT/mTOR signature appeared to be necessary for the survival of TEL-AML1 leukaemia (Fuka et al., 2012). These findings suggest that the fusion gene may also have an active role in upregulating the expression of genes fundamental to the activity of oncogenic pathways and not only function as an antagonist of wild-type RUNX1. Identifying and characterising the transcriptional cascades and signalling pathways deregulated by TEL-AML1 is therefore essential to understanding how this fusion protein promotes leukaemia in children.

1.3 Aim of the study: Cancer signalling and targeted therapy

Most cancers are multistep processes of malignant transformation that require activation or down-regulation of several cellular processes. A defect in every process is often associated with alteration of a particular transcription or signalling pathway in the cell that can alter normal programs of proliferation, transcription, growth, migration, differentiation and death. In the last 10 years the identification of these pathways has allowed the discovery of new drug targets for cancer treatment, specific for many different tumours subtypes. Moreover, considerable attention has been directed towards the identification of the oncoproteins necessary for particular cancers, in order to target the specific factor or pathways affected with a selected drug (oncogene addiction) (Weinstein, 2002). For example, impressive results have been obtained in treatment of selected cohorts of patients harbouring oncogene-addicted tumours. The drug Imatinib (Gleevec), an inhibitor of the tyrosine kinase activity of the BCR-ABL fusion protein, has shown particular success in therapy of chronic myeloid leukaemia (CML) and and gastro-intestinal stromal tumours (GIST) (Goldman and Marin, 2012; Pandey and Kochar, 2012). Other examples are Trastuzumab, that targets the HER2/neu receptor in breast cancer, Erlotinib and Gefitinib, two epidermal growth factor receptor (EGFR) inhibitors, in non-small cell lung cancer (NSCLC) (Torti and Trusolino, 2011). However, this approach is not valid for all the patients and frequently the

benefit is of limited duration. A possible partial explanation could be found in the heterogeneity of the cancer, especially in solid tumours. The presence of low-frequency multiple sub-populations that are not dependent on a driver event may, in fact, significantly influence treatment outcome in many solid tumours (Mendelsohn, 2013; Parsons and Myers, 2013). Tumour heterogeneity has also been found to occur in TEL-AML1 leukaemia (Anderson et al., 2011). Analysis of the clones, however, showed that independent of the clonal evolution and its architectural complexity, the *TEL-AML1* fusion gene was present in all the sub-clones, suggesting that these may be highly dependent on the activity of this single oncogene for continued cell proliferation and survival (Figure 1-10). However, transcription factors are historically avoided as therapeutic targets because of the difficulty of developing drugs that interfere with their binding to DNA and the associated difficulty to selectively target nuclear biochemical events (Darnell, 2002; Konstantinopoulos and Papavassiliou, 2011). Furthermore, even if such targeting was possible, inhibition of transcription factor function may result in unwanted toxicity. For example, targeting TEL or AML1 may have disrupting effects on the HSCs compartment completely compromising haematopoiesis. From this perspective understanding the genes and pathways controlled directly by TEL-AML1 could help to identify new therapeutic targets that can be used for the treatment of this particular leukaemia. Indeed the aim of the present study was to identify specific signalling pathways regulated by the fusion gene necessary for the survival of TEL-AML1 leukaemia. In order to achieve this we used two different

models: mouse haematopoietic progenitor cells, in order to study the effect of overexpression of the fusion gene, and human leukaemic cell lines carrying the t(12;21) translocation to confirm the findings obtained with the mouse model, with the use of commercially available selective inhibitors or/and using RNA interference methodology.

1.4 Figures

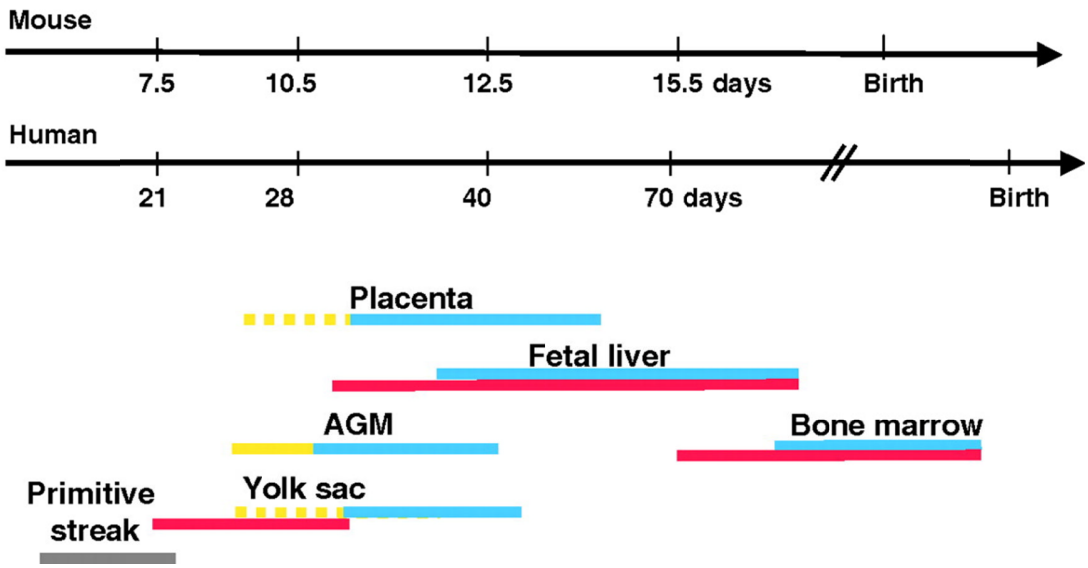


Figure 1-1: Establishment of primitive definitive haematopoiesis in mouse and human embryos [adapted from (Mikkola and Orkin, 2006)].

The figure shows haematopoiesis locations in mouse and in human at different age stages. Red bars represent active haematopoietic differentiation, yellow bars represent generation of HSCs and blue bars represent adult and foetal HSCs. Broken yellow bars represent putative *de novo* generation of HSCs that has not been experimentally confirmed.

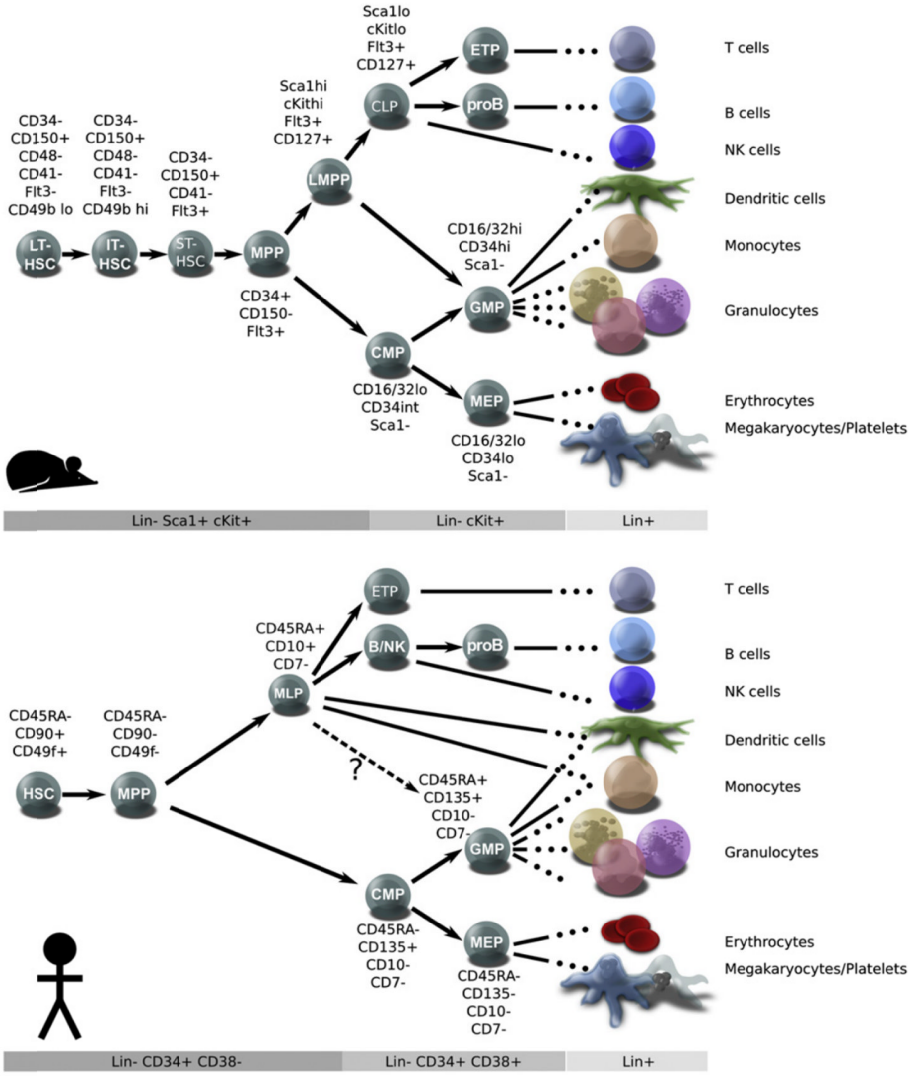


Figure 1-2: Current model of mouse and human haematopoiesis [Adapted from (Doulatov et al., 2012)].

The diagrams illustrate the current model for mouse and human haematopoieis. Stem cells and progenitor cell are mainly defined by cell surface markers, which are listed for every population. In mouse, HSCs can be separated into long-term (LT), intermediate-term (IT), and short-term (ST) classes based on the duration of repopulation. Differentiation of these populations give rise to engrafting multipotent progenitors (MPPs), and a series

of immature lymphoid-biased progenitors (such as LMPPs) that undergo gradual lymphoid specification. In human, HSCs generate MPPs. Contrary to what has been described in mice only one population of immature lymphoid progenitors (MLPs) has been identified. Terminally differentiated cells are shown on the right, and inferred lineage relationships are depicted with arrows.

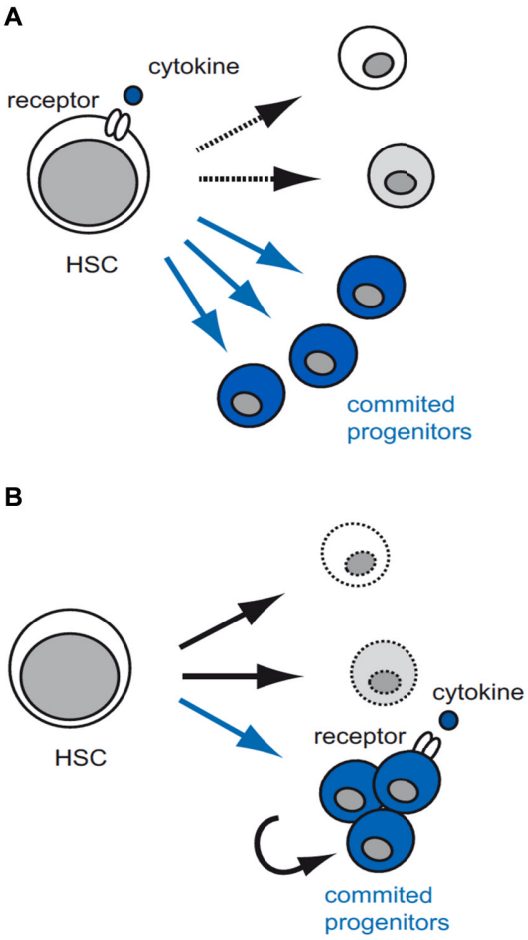


Figure 1-3: Function of cytokines in the instructive versus the stochastic model of haematopoiesis [Adapted from (Sarrazin and Sieweke, 2011)].

The diagrams show the different action of cytokines according to the two models of haematopoiesis. In the instructive model (**A**) cytokines define commitment of a stem cell to a specific lineage leading to increased production of specific committed progenitors. In the stochastic model (**B**), commitment is cytokine independent and the function of these factors is to support the selective survival or proliferation of committed cells.

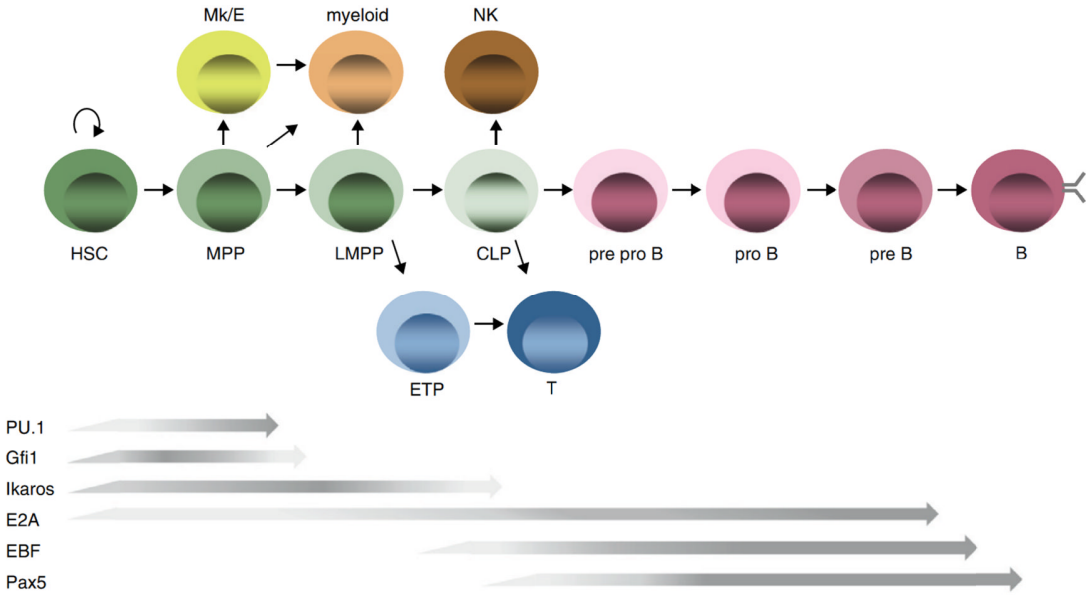


Figure 1-4: B cell differentiation from HSC [Adapted from (Santos and Borghesi, 2011)].

The diagram illustrates the model of B cell development from HSCs. This model demonstrates progression to multipotent progenitor (MPP), lymphoid/myeloid-primed multipotent progenitor (LMPP), common lymphoid progenitor (CLP) and B cell progenitor intermediates before B cell commitment. Establishment of B cell fate is also accompanied by the loss of expression of genes involved in self-renewal and myeloid commitment and increased expression of B lineage specific genes.

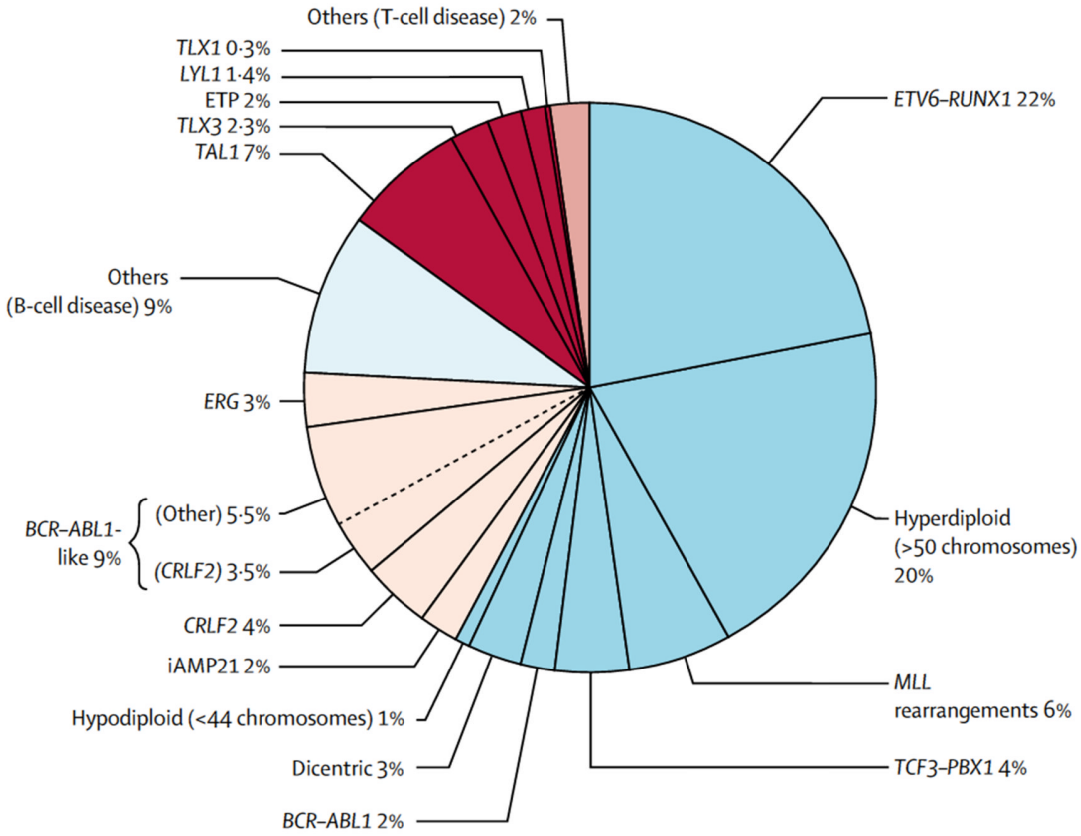


Figure 1-5: Frequency of abnormalities in paediatric Acute Lymphoblastic Leukaemia [Adapted from (Inaba et al., 2013)].

The chart shows the cytogenetic and genetic alteration with the relative frequencies present in paediatric ALL. The most frequent is represented by the t(12;21) translocation.

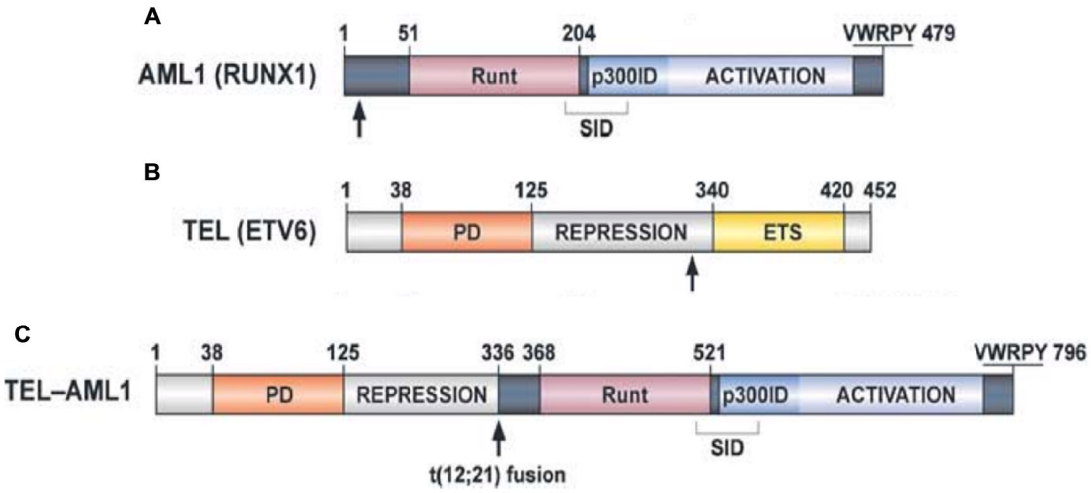


Figure 1-6: schematic representation of AML1, TEL and the relative fusion protein [Adapted from (Zelent et al., 2004)].

The figure shows a schematic representation of the full-length AML1 (**A**), TEL (**B**) and TEL-AML1 (**C**) proteins. AML1 is characterized by the presence of the RUNT domain, necessary to mediate DNA binding, the p300HAT (p300ID) interaction domain, the mSin3A interaction domain (SID) and the C-terminus domain necessary for protein-protein interactions. TEL has three main domains: the pointed domain (PD) and the central repression domain for protein-protein interaction and the ETS domain for DNA binding. The TEL-AML1 fusion protein contains almost the entire full length protein of AML1 and the PD and repression domains of TEL. The fusion points between TEL and AML1 are indicated by arrows.

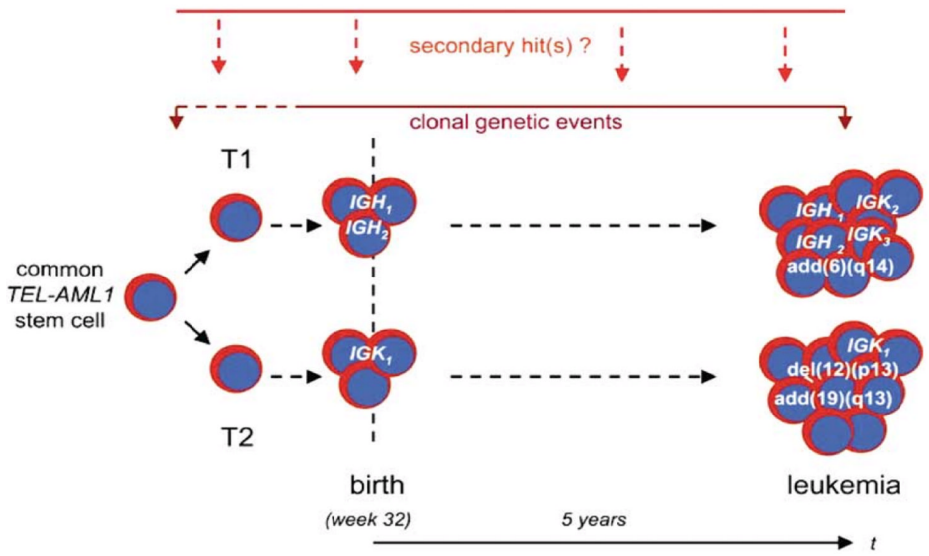


Figure 1-7: TEL-AML1^{+} leukaemia is a multistep disease [Adapted from (Teuffel et al., 2004)].

The diagram illustrates the hypothesised requirement for secondary genetic alterations in TEL-AML1 leukaemia, from analyses in monozygotic twins. It indicates that immunoglobulin gene rearrangements and secondary genetic alterations in leukaemic cells can arise independently from the generation of a common TEL-AML1 stem cell.

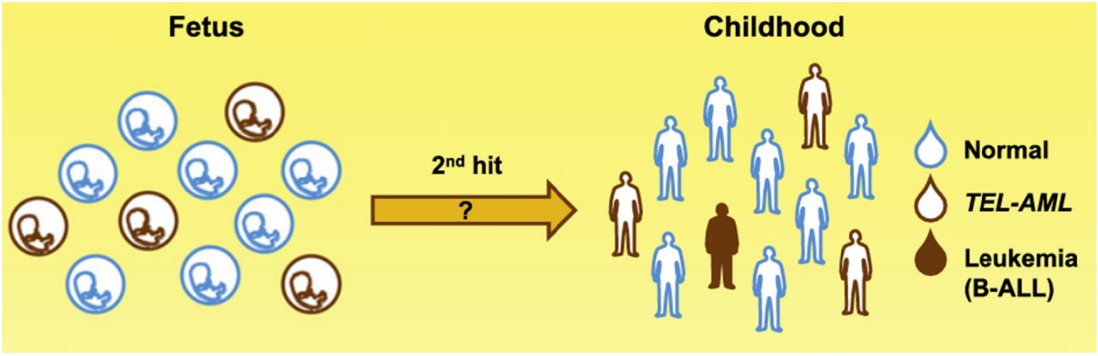


Figure 1-8: Second hit model in TEL-AML1 leukaemia. [Adapted from (Sloma and Eaves, 2009)].

The diagram illustrates the hypothesis that the presence of the TEL-AML1 fusion *per se* is not sufficient for malignant transformation (as shown in Figure 1-7). The frequency of t(12;21) is significantly higher in normal newborns in comparison to the frequency of the disease. This data is confirmed by the fact the adults can still carry the translocation without any signs of disease.

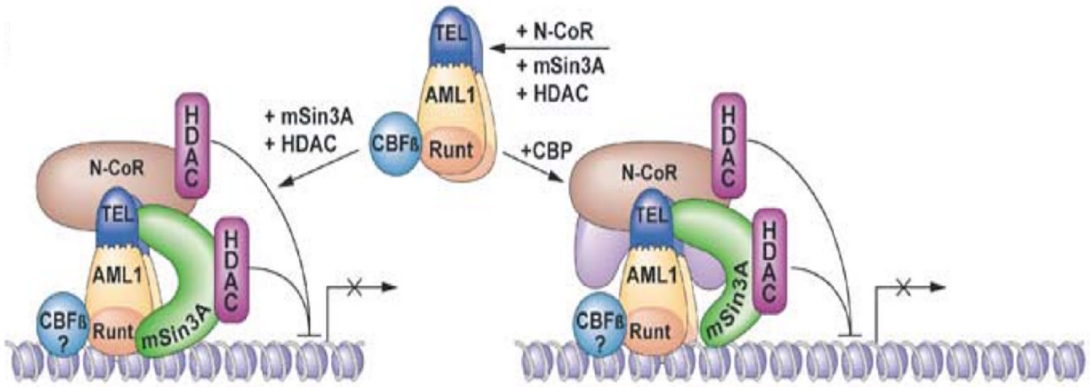


Figure 1-9: Repression model of TEL-AML1 function [Adapted from (Zelent et al., 2004)].

The diagram shows the hypothetical model of the molecular function of TEL-AML1. According to this model the fusion protein works as a constitutive transcriptional repressor. This could be due to the ability of TEL (ETV6) to bind corepressor complexes such as mSin3A and N-CoR. Moreover, AML1 (RUNX1) retains the ability to bind HDACs but loses the capacity to bind Histone acetyltransferase (HAT) proteins. Since the fusion protein preserves the DNA binding domain of AML1 it is widely believed that the chimeric protein mainly acts as negative regulator of the wild type activity of AML1.

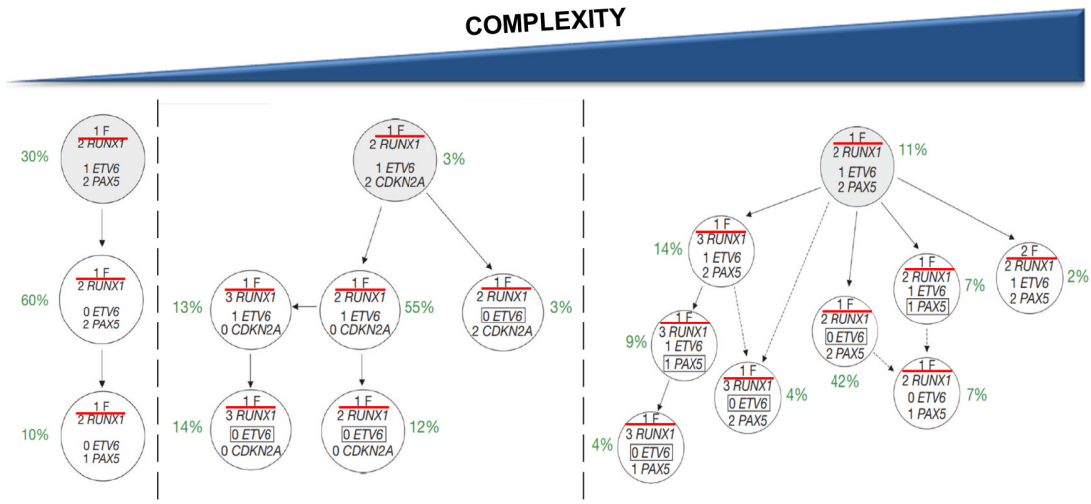


Figure 1-10: Heterogeneity in TEL-AML1 leukaemia. [Adapted from (Anderson et al., 2011)].

The diagram illustrates three examples of different sub-clonal architectures analysed in three TEL-AML1^{+} leukaemia patients. This architecture varies from very simple (left) to very complex (right), carrying different genetic alterations in all the sub-clones. However, independent of this complexity, all the sub-clones contain the fusion gene specified with F. Every sub-clone is represented by a circle, inside of which the different types of secondary genetic alterations are indicated.

CHAPTER II

MATERIALS AND METHODS

2.1 Molecular biology

2.1.1 Transformation of bacteria

Sub-cloning efficiency DH5α™, library efficiency DH5α™ and One Shot® Stbl3™ chemically competent cells (Life Technologies – Invitrogen) were used for transforming bacteria. At least 0.5µg of DNA was incubated in pre-chilled polypropylene tubes (BD Bioscience) together with the competent cells, previously thawed on ice. The mixture was incubated on ice for 30 minutes, followed by heat shock for 30 seconds in a 37°C waterbath. The mixture was subsequently incubated on ice for 2 minutes and then 300µL of SOC outgrowth medium (New England BioLabs) was added. The mixture was then incubated in a shaker at 37°C for 1 hour and then plated onto plates containing LB agar (1.5g bacto Agar (BD Bioscience) per 100ml LB broth (1% w/v Bacto Tryptone (BD Bioscience), 0.5% w/v bacto Yeast Extract (BD Bioscience), 1% w/v Sodium Chloride (NaCl), [pH 7.0]) and 100µg/ml Ampicillin (Sigma-Aldrich) or 100µg/ml Carbenicillin (Sigma-Aldrich) and incubated at 37°C overnight.

2.1.2 Isolation of plasmid DNA

Individual bacterial colonies were inoculated into 3ml LB broth containing the antibiotic for the selection and incubated in a shaker at 37°C overnight. The

bacterial cultures were then used to extract DNA using the PureYield™ Plasmid Miniprep System (Promega) according to the manufacturer's instructions. 100µl of lysis buffer was added to 600µl of bacterial culture. After inverting the tubes 350µl of cold neutralization buffer was added and the mixture centrifuged in a microcentrifuge at maximum speed for 3 minutes (16000g). The supernatant was transferred to a column and centrifuged at maximum speed for 15 seconds and the flow-through discarded. The column was washed first with endotoxin removal buffer then with the column wash solution containing ethanol. The plasmid DNA was eluted with 30µl of distilled water. The DNA concentration was measured using a spectrophotometer (NanoDrop.ND-1000, Lebtech International).

In order to obtain large quantities of plasmid DNA individual bacterial colonies were inoculated into 3ml LB broth containing the antibiotic for selection and incubated in a shaker at 37℃ for 6 hours. This culture was then added to 300ml LB broth broth containing the antibiotic for selection and incubated in a shaker at 37℃ overnight. The genopure plasmid maxi kit (Roche) was used to isolate the plasmid DNA according to manufacturer's guidelines. The bacterial culture was centrifuged for 20 minutes at 16000g. The pellet was resuspended with 24ml for lentiviral vector DNA or 12ml for normal plasmid of resuspension buffer and next the same volume of lysis buffer was added and incubated for 2-3 minutes at room temperature. 12 ml of chilled neutralisation buffer was added to the mixture, the tubes inverted 10 times and incubated for 5 minutes on ice. The lysate was cleared by filtration and loaded onto a pre-equilibrated column.

The column was washed 3 times with 16ml of wash buffer then eluted with pre-warmed 15ml elution buffer. The eluted plasmid DNA was precipitated with 11ml of isopropanol and centrifuged at 4000g for 1 hour. The pellet was washed with 70% ethanol, centrifuged for 10 minutes and air-dried. The DNA plasmid was re-dissolved in distilled water and the concentration measured with the NanoDrop.

2.1.3 Restriction enzyme digests

Restriction enzyme digests were performed according to the manufacturer's instructions. In general DNA was digested with 0.3µl or 0.5 µl of 10U/µl restriction enzyme per µg, 10x restriction buffer and 100x BSA 10µg/µl and the volume made up to 200µl with H_20. The DNA was digested for 1-4 hours depending on the restriction enzyme used. Depending on the size of the fragment, the digested products were subjected to electrophoresis on 0.7-2% w/v agarose gels [agarose (Invitrogen), 1x TAE buffer (National diagnostic), 0.5% ethidium bromide (Sigma-Aldrich)], in order to be visualised.

In order to generate fragmented DNA with a blunt end for some of the cloning procedures, T4 DNA polymerase (Promega) was used according to the manufacturer's protocol. In general, 2µg of fragmented DNA was filled in with 10U of T4 DNA polymerase, 100µM of each dNTP, 10µl of 10x reaction buffer [250mM Tris-Acetate (pH 7.7), 1M potassium acetate, 100mM magnesium acetate and 10mM DTT] and H_20 was added to make the final volume 100µl.

The mixture was incubated for 30 minutes at 37℃ and 4µl of 0.5M Ethylenediaminetetraacetic acid (EDTA) was then added to stop the reaction.

2.1.4 Gel extraction

Isolated digested DNA products were purified from the agarose gel using QIAquick Nucleotide removal kit (Qiagen) according to the manufacturer's guidelines. The DNA fragment was excised from the agarose gel and weighed. A volume corresponding to three times this weight of buffer QG was added to the excised gel and incubated at 50℃ for 10 minutes, or until the gel was completely dissolved. An equal volume corresponding to the weight of the gel of isopropanol was added and the mixture was transferred onto a QIAquick spin column and centrifuged at maximum speed for 1 minute. The column was washed to remove any salt contamination with 0.7ml of buffer PE for 1 minute at maximum speed, emptied and centrifuged for 1 additional minute to completely remove ethanol contamination. DNA was then eluted by centrifuging the column with 30µl of elution buffet.

2.1.5 Ligation

The DNA fragment and vector were ligated to generate plasmid DNA. Depending on the cloning, the molar ratio between the fragment and the vector varied from 1:1 to 5:1. Generally, 50ng of the vector were ligated with the required amount of DNA fragment using 5µl of 2x Rapid Ligation Buffer [60mM

Tris-HCl (pH 7.8), 20mM MgCl2, 20mM DTT, 2mM ATP and 10% PEG] (Promega) and 1 weiss unit of T4 DNA ligase (Promega), in a total volume of 10µl, made up with H_2O. The mixture was incubated at room temperature for 15 minutes prior to transformation (see section 2.1).

2.1.6 DNA constructs

2.1.6.1 Retroviral vector

A schematic diagram of the retroviral expression constructs is shown in Figure 2.1. TEL-AML1 mutant carries two point mutations in AML1 sequence (Y140A/T188A). This mutant is not able to bind the partner protein Core-binding factor subunit beta (CBFbeta) but can still bind DNA (Roudaia et al., 2009).

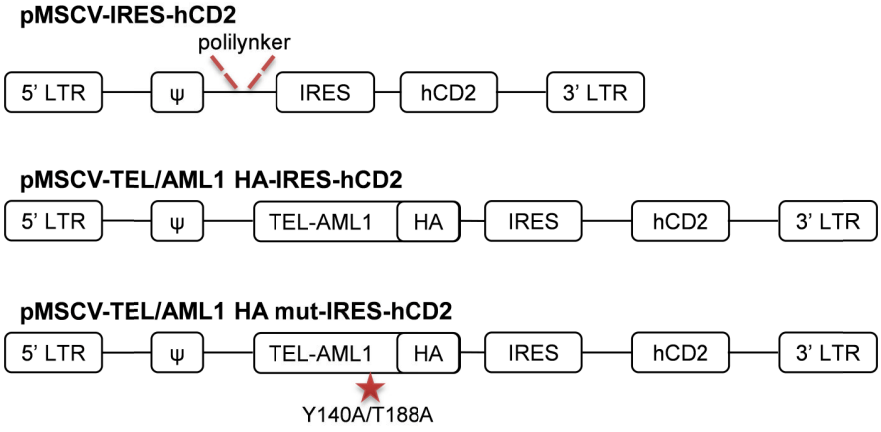


Figure 2-1: Retroviral expression vectors used in this study.

LTR, long terminal repeat; ψ, viral packaging signal; HA, hemagglutinin tag; IRES, internal ribosome entry site; hCD2, human CD2.

2.1.6.2 Lentiviral vector

Schematic diagram of the lentiviral expression constructs is shown in Figure 2.2. The pHR-SIN-CSGW was a kind gift from gift from Y.Ikeda (Mayo Clinic, Rochester, MN). This vector was obtained removing one NotI site from the SIN-CSGW vector. The vector was modified replacing the eGFP sequence with a PGK-PURO-IRES-tGFP cloned from a pMSCV-PGK-PURO-IRES-eGFP.

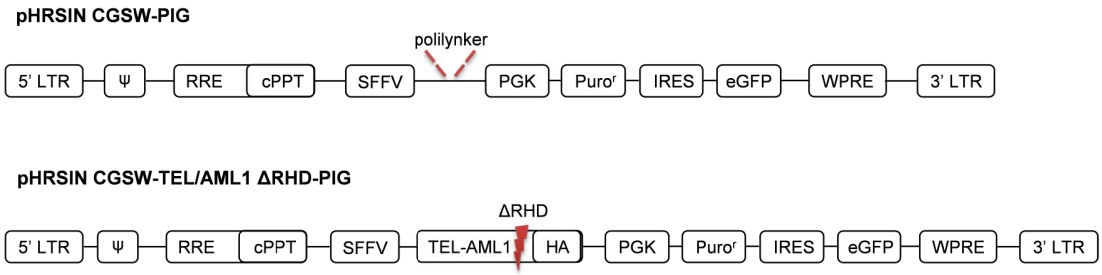


Figure 2-2: Lentiviral expression vectors used in this study.

LTR, long terminal repeat; ψ, viral packaging signal; RRE: Rev response element; cPPT, central polypurine tract; SFFV, spleen focus-forming virus promoter; ΔRHD, delition of the Runt homology domain; PGK, phosphoglycerate kinase promoter; eGFP, enhanced green fluorescent protein; Puro^r, puromycin resistance; IRES, internal ribosome entry site; WPRE, woodchuck hepatitis virus regulatory element

2.1.6.3 Small hairpin RNA (shRNA) vector

Lentiviral MISSION shRNA constructs targeting *TEL* (Clone ID: NM_001987.x-309s1c1), *STAT3* (Clone ID: NM_003150.3-1242s21c1 [shSTAT3_1] and

NM_003150.2-458s1c1 [shSTAT3_2]), *MYC* (Clone ID: NM_002467.2-1377s1c1 [shMYC_1] and NM_002467.2-1377s1c2 [shMYC_2]) and non-silencing control (SHC002) were purchased from Sigma-Aldrich (Figure 2-3). The *TEL*-specific shRNA was used to specifically silence *TEL-AML1* fusion gene expression in Reh cells, since the wild-type *TEL* allele is deleted in this cell line.

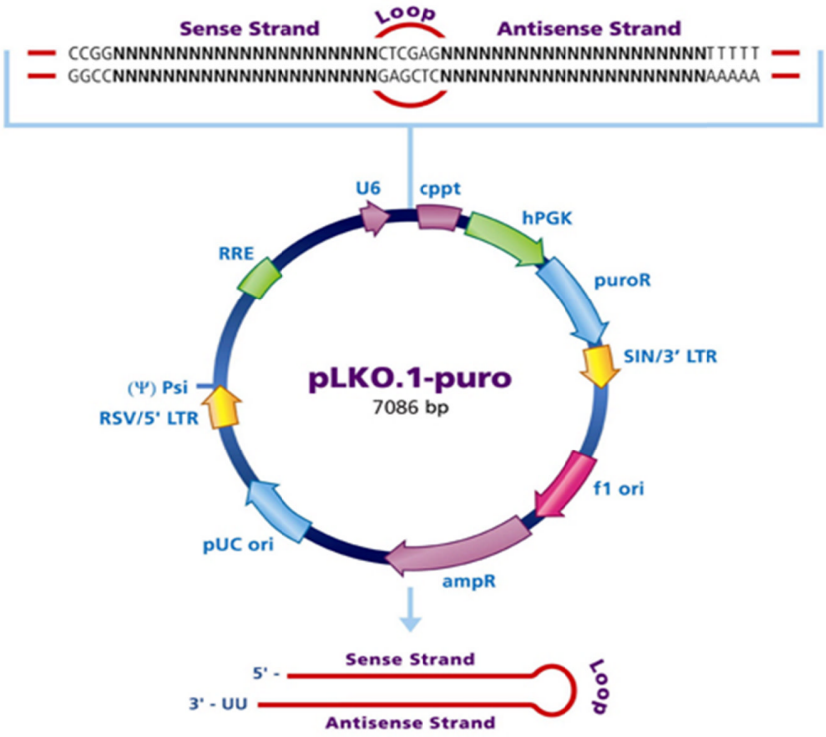


Figure 2-3: Graphic representation of the shRNA vector used in this study [Adapted from (http://www.sigmaaldrich.com/life-science/functional-genomics-and-rnai/shrna/library-information/vector-map.html)].

The diagram represents the graphical map of the shRNA vector used in this study. The human U6 promoter (a pol III promoter) is used to drive expression of the shRNA hairpin, whereas the Human phosphoglycerate kinase eukaryotic promoter (hPGK) promoter drives the expression of the Puromycin resistance

gene for mammalian selection (puroR). ccpt, central polypurine tract; SIN/LTR, 3' self-inactivating long terminal repeat; f1 ori, f1 origin of replication; ampR: ampicillin resistance gene for bacterial selection; pUC ori, pUC origin of replication; 5' LTR, 5' long terminal repeat; Psi, RNA packaging signal; RRE, Rev response element.

GENE	sequence (5'-3') (sense-loop-antisense)
Non-Mammalian shRNA Control	CCGG CAACAAGATGAAGAGCACCAA CTCGAG TTGGTGCTCTTCATCTTGTTG TTTTT
ETV6 **(NM_001987)**	CCGG GCTGCTGACCAAAGAGGACTT CTCGAG AAGTCCTCTTTGGTCAGCAGC TTTTT
STAT3_1 **(NM_003150)**	CCGG GGCGTCCAGTTCACTACTAAA CTCGAG TTTAGTAGTGAACTGGACGCC TTTTTG
STAT3_2 **(NM_003150)**	CCGG GCACAATCTACGAAGAATCAA CTCGAG TTGATTCTTCGTAGATTGTGC TTTTT
MYC_1 **(NM_002467)**	CCGG CCTGAGACAGATCAGCAACAA CTCGAG TTGTTGCTGATCTGTCTCAGG TTTTTG
MYC_2 **(NM_002467)**	CCGG CCTGAGACAGATCAGCAACAA CTCGAG TTGTTGCTGATCTGTCTCAGG TTTTTG
ARHGEF4 **(NM_015320)**	CCGG CCTCCATGTGAGCATCAAGAA CTCGAG TTCTTGATGCTCACATGGAGG TTTTTG

Table 1: shRNA sequences used in this study.

2.1.7 Preparation of total protein lysate for western blot analysis

Cells were harvested and washed with PBS by centrifugation at 300g for 5 minutes at 4ºC. Cell pellets were lysed using 60µL of 1x RIPA (Radio-Immunoprecipitation Assay) buffer (Cell Signaling) [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin] per $1x10^6$ cells. The lysate was incubated for 20 minutes in ice, vortexed for 10 seconds, sonicated for 10 seconds and centrifugated at 16,000g for 20 minutes at 4°C. The supernatant was collected and the total cell lysate was stored at -80°C.

For nuclear and cytoplasmic extracts the Nuclear Extract Kit (Active Motif) was used according to the manufacturer's instructions. In brief, at least $1x10^7$ cells were harvested and washed with phosphate buffered saline (PBS) by centrifugation at 300g for 5 minutes at 4ºC. Cell pellets were lysed using 500µl 1X hypotonic buffer and incubated for 15 minutes on ice. 25 µl of detergent was added to the lysate and vigorously vortexed for 10 seconds. The suspension was centrifuged for 30 seconds at 14,000 x g in a microcentrifuge, pre-cooled at 4ºC and the supernatant representing the cytoplasmic fraction was collected and stored at -80°C. The nuclear pellet was resuspend in 50 µl complete lysis buffer, vigorously vortexed for 10 seconds and incubated for 30 minutes on ice. The nuclear lysate was then centrifuged for 10 minutes at 14,000g in a microcentrifuge pre-cooled at 4ºC and the supernatant (nuclear fraction) was collected into a pre-chilled microcentrifuge tube and stored at -80°C.

Protein concentration was measured using the Bradford reagent (BioRad), by measuring the absorbance at a wavelength of 595nm using the Ultrospec 2100 pro (Amersham Pharmacia Biotech) spectrophotometer.

The NuPAGE system from Invitrogen was used to perform protein gel electrophoresis (NuPAGE® Novex® 10% Bis-Tris Gels were used for most of the analyses, whereas NuPAGE® Novex® 8% Bis-Tris Gels were used for TEL-AML1 analysis and NuPAGE® Novex® 4-12% Bis-Tris Gels were used for RAC1 analysis). Following electrophoresis, samples were transferred onto a polyvinylidenefluoride (PVDF) membrane (Millipore) for 3 hours at 400 mA at 4ºC in a Tris-glycine-methanol transfer buffer [25mM Tris (Sigma-Aldrich), 192mM glycine (Sigma-Aldrich), 20% methanol].

2.1.8 Western blot analysis

Membranes were blocked in PBS with 5% non-fat milk and 0.2% Tween-20, and stained with one the antibodies listed in Table 2. Proteins were detected using the secondary antibodies conjugated with horseradish peroxidise listed in Table 3 and a chemiluminescent reagent (ECL, GE Healthcare) or SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific), according to the manufacturer's instructions. The membrane was stripped using Restore™ Western Blot Stripping Buffer (Thermo Scientific) for some experiments for 10 minutes at room temperature and re-probed with different primary and secondary antibodies.

Exposed film was developed using the Xograph CompactX4 (BioRad) developer. The film was then acquired using a calibrated densitometer (GS-800, BioRad) and individual bands were quantified using QuantityOne software (BioRad). The relative protein expression was obtained by dividing the density values of the protein of interest by the density values of the appropriate loading control.

Name of antibodies	Supplier	Dilution
STAT3 (clone 79D7)	Cell Signaling Technology	1:1000
STAT3 Y705	Cell Signaling Technology	1:500
STAT3 S727	Cell Signaling Technology	1:500
GAPDH (v-18)	Santa Cruz Biotechnology	1:1000
HSP90 (F-8)	Santa Cruz Biotechnology	1:1000
TEL (N-19)	Santa Cruz Biotechnology	1:500
TEL (C-214)	Santa Cruz Biotechnology	1:500
HLH-TEL	Kind gift of Jan Cools (University of Leuven, Belgium)	1:1000
cleaved CASPASE 3 (5A1)	Cell Signaling Technology	1:500
MYC (9E10)	Santa Cruz Biotechnology	1:500
p21 (F-5)	Santa Cruz Biotechnology	1:500
p27 (C-19)	Santa Cruz Biotechnology	1:500
LAMIN B	Santa Cruz Biotechnology	1:1000
RAC1	Thermo Scientific	1:1000
SURVIVIN (D-8)	Santa Cruz Biotechnology	1:1000
AML1	Santa Cruz Biotechnology	1:500

Table 2: Primary antibodies used for western blot analyses in this study.

Name of antibodies	Supplier	Dilution
anti-mouse IgG HPR-linked whole antibody	GE HealthCare	1:3000
anti-rabbit IgG HPR-linked whole antibody	GE HealthCare	1:5000
anti-goat IgG HPR-linked whole antibody	Santa Cruz Biotechnology	1:5000

Table 3: Secondary antibodies used for western blot analyses in this study.

2.1.9 RAC1-GTP pull down

RAC1 activity was detected by measuring the quantity of RAC1-GTP complex in the cells using the Active Rac1 Pull-Down and Detection Kit according to manufacturer's instructions (Thermo Scientific). Cells were washed with ice-cold tris-buffered saline (TBS) and the pellet was resuspended with 500µl Lysis/Binding/Wash Buffer [100mL, 25mM Tris•HCl, pH 7.2, 150mM NaCl, 5mM MgCl2, 1% NP-40 and 5% glycerol] and incubated on ice for 5 minutes. The lysate was centrifuged at 16000g for 15 minutes. After protein quantification at least 500µg of total protein was transferred onto a column pre-washed with 400µl Lysis/Binding/Wash Buffer and pre-loaded with glutathione resin. 20µg of GST-human PAK1-PBD was also added to the column. The reaction mixture was incubated at 4°C for 1 hour with gentle rocking and then the column was washed three times with 400µl Lysis/Binding/Wash Buffer. The column was eluted with 50µl of 2X SDS Sample Buffer [1.5mL, 125mM

Tris•HCl, pH 6.8, 2% glycerol, 4% SDS (w/v) and 0.05% bromophenol blue] with β-mercaptoethanol and stored at -20℃.

2.1.10 RNA isolation, cDNA preparation and quantitative Real-Time PCR

Total RNA was isolated from the cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The cells were disrupted by adding buffer RLT, containing β-mercaptoethanol. Homogenization of the samples was achieved by shearing of genomic DNA using a 18G needle. One volume of 70% ethanol was added to the samples and the mixture was transferred into a RNeasy spin column. After centrifugation the column was washed with 700µl buffer RW1 first and then subsequently with 500µl buffer RPE. The RNA was eluted with 30µl of diethylpyrocarbonate (DEPC) treated water and the concentration determined using a spectrophotometer (NanoDrop ND-1000, Lebtech International).

RNA was converted into cDNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems) according to the manufacturer's instructions. 1µg of RNA was converted using 1µl 20X Enzyme mix and 5µl 2X RT Buffer Mix in a total volume of 10µl. Samples were treated with DNase (Invitrogen) prior the reverse transcription.

Quantitative RT-PCR (qRT-PCR) was performed on isolated mRNA using TaqMan probe based chemistry and an ABI Prism 7900HT fast Sequence

Detection System (Life Technologies). All primer/probe sets were from Applied Biosystems, Life Technologies except for *TEL* mRNA detection, for which a previously published primer/probe set was used (Taube et al., 2004).

2.2 Cell biology

2.2.1 Cell culture and cell lines

The LinXE [Genetica, (Hannon, 2002)] and 293FT (Invitrogen) packaging cell lines were cultured in Dulbecco's Modified Eagle's medium (DMEM, Invitrogen Paisley) supplemented with 10% heat-inactivated foetal calf serum (FCS, Sigma-Aldrich), 100U/ml penicillin (Invitrogen), 100µg/ml streptomycin (Invitrogen) and 2mM L-glutamine (Invitrogen) (complete DMEM). LinXE cells were maintained with 7.5µg/ml hygromycin (Invivogen). Human leukaemic cell lines (Table 4) were cultured in Roswell Park Memorial Institute (RPMI) medium (Invitrogen) supplemented with 10% heat-inactivated FCS, 100U/ml penicillin (Invivogen), 100µg/ml streptomycin (Invitrogen) and 2mM L-glutamine (complete RPMI). Mouse c-kit^{+}Ter119^{-} foetal liver haematopoietic progenitor cells were cultured in complete DMEM medium supplemented with 100ng/ml stem cell factor (SCF), 10ng/ml interleukin-7 (IL-7), 10ng/ml Fms-like tyrosine kinase 3 (FLT-3) (all from Peprotech) and 50µM 2-mercaptoehanol (2-ME) (Sigma-Aldrich). The human leukaemic cell lines Reh, 697, Tom-I and Sup-B15 were obtained from the German Collection of Microorgamisms and Cell Cultures (DSMZ, Brunswick, Germany). Bel-I and Semk were the kind gift of R.

Stam (Erasmus MC, Rotterdam, the Netherlands), and At-2 were the kind gift of R. Panzer-Grümayer (Children's Cancer Research Institute, Vienna, Austria). Each cell line was cultured at between 0.2-0.8x10^6/ml and sub-cultured every three to four days according to the supplier's instructions (DSMZ).

Cell line	Translocation	Fusion gene
Reh	t(12;21)	TEL-AML1
At-2	t(12;21)	TEL-AML1
Tom-1	t(9;22)	BCR-ABL
Sup-B15	t(9;22)	BCR-ABL
Rs4;11	t(4;11)	MLL-AF4
697	t(1;19)	E2A-PBX1
Bel-1	t(4;11)	MLL-AF4
Semk-2	t(4;11)	MLL-AF4

Table 4: Human B-ALL cell lines used in this study.

2.2.2 Purification of haematopoietic progenitor cells

c-Kit^+ $Ter119^-$ hematopoietic progenitor cells (HPCs) were purified from embryonic day 12 (E12) foetal liver of C57BL/6 mice by magnetic bead cell sorting (Miltenyi Biotec). The livers were carefully isolated and after red cell lysis cells were stained with Ter119 PE antibody (BD Bioscence), washed and incubated with 20µl anti-PE microbeads for 20 minutes at 4°C. $TER119^-$ cells were isolated by magnetic separation using column type LS (Miltenyi Biotec). The effluent was collected as the negative fraction, washed and stained with

the c-Kit antibody (Biolegend). The cells were then incubated with 20µl anti-APC micro-beads for 20 minutes in the fridge. c-Kit$^+$ cells were isolated by magnetic separation using column type MS (Miltenyi Biotec), washed and resuspended in appropriate DMEM complete medium

2.2.3 Retroviral and lentiviral packaging cell line transfection

LinXE cells were seeded without hygromycin at a density of 0.75x10^6 per 10cm petri dish (Thermo Fisher Scientific, Leicestershire, UK) three days before the transfection for the production of retrovirus. A total of 8µg of expression vector was incubated in 1.5ml Optimem (GIBCO) plus 36µl Lipofectamine 2000 reagent (Invitrogen) for 20 minutes at room temperature and then the DNA-Lipofectamine 2000 complexes were added to the cells. Lipofectamine containing medium was then replaced with 10ml of complete DMEM medium on the second day of transfection.

For lentiviral packaging cell line transfection, 293FT cells were seeded at a density of 0.5x10^6 per 10cm petri dish three days before transfection for the production of lentivirus. For lentiviral transfection, 5µg of expression vector, 3.75µg of pCMV-PAX2 (which was kindly supplied by Professor D. Trono, Lausanne, Switzerland) and 1.5µg of pVSV-G construct were incubated in 1ml Optimem (GIBCO) plus 30µl MegaTran (OriGene) reagent for 10 minutes at room temperature and then the DNA-MegaTran complexes were added to the

cells. MegaTran containing medium was then replaced with 10ml of complete DMEM medium on the second day of transfection.

2.2.4 Retroviral transduction of murine cell lines and lentiviral transduction of human leukaemic cell lines

Retroviral supernatant was collected 48 hours after the transfection and concentrated 2-fold for some experiments. The unfiltered supernatant was cleared of cell debris by two rounds of centrifugation at 580g for 5 minutes. The cleared virus was then aliquotted into 1.5ml eppendorf tubes and concentrated by centrifugation using a micro-centrifuge at 16,000g for one hour at 4ºC. Mouse c-Kit^{+}Ter119^{-} foetal liver haematopoietic progenitor cells were seeded at 1x10^{4} cells per well, supplemented with 20% FCS, 100ng/ml SCF, 10ng/ml IL-7, 10ng/ml FLT-3 and 5µg/ml polybrene (Sigma-Aldrich) and transduced in 96-well flat bottomed plates (Thermo Fisher Scientific) by spinoculation (centrifugation at 700g, for 45 minutes at 25 ºC). 24 hours after transduction, 100µl per well of complete DMEM with growth factors 100ng/ml SCF, 10ng/ml IL-7, 10ng/ml FLT-3 was added. The transduced murine cells were used for further experiments after 48, 72 or 96 hours of transduction.

For lentiviral transduction of Reh and AT-2 cells, 293FT supernatant was cleared using Minisart 0.45µm filters (Sartorius Stedim Biotech). Ultracentrifugation was performed to concentrate lentivirus in some experiments. Sorvall 12ml centrifuge tubes (Thermo Scientific) were sterilized

with 70% ethanol and washed twice with PBS prior to use. 11.5ml of filtered virus was added to each tube and concentrated by ultracentrifugation at 18000g for 3 hours at 4°C (Thermo Scientific, Discovery 100). The supernatant was discarded and the pellet was resuspended in 1.5ml of RPMI medium. $0.5x10^5$ cells supplemented with 5µg/ml polybrene in 96-well plates, were transduced by spinoculation at 700g, for 45 minutes at 25 ºC. 24 hours after the transduction, 100µl per well of complete RPMI was added. 48 hours after the transduction the cells were harvested and plated in complete RPMI supplemented of 0.6µg/mL puromycin depending on the lentiviral vector used.

2.2.5 Primary patient samples

Informed consent was given by parents and/or guardians to use excess leukaemic cell material remaining after diagnostic procedures, for research purposes as approved by the Institutional Review Board of the Erasmus MC. Patient sample purification procedures and proliferation analysis was performed in Professor M. Den Boer's Laboratory (Erasmus Mc, Rotterdam). Mononuclear cells were separated by sucrose gradient centrifugation (Lymphoprep 1.077g/ml density; Nycomed Pharma) and mononuclear cells were collected for further processing. The percentage of leukemic cells was increased >90% by eliminating contaminating normal lymphocytes and myeloid progenitor cells using antibody-coated beads as previously described (Den Boer et al., 2003). Cells were exposed to a 2-fold serial dilution series of STAT3 inhibitor for 96 hours at 37°C in humidified air containing 5% CO_2. Next,

cells were incubated with 0.33µg/µl tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS; Promega) and 7.5µg/ml of the electron coupling reagent phenazine methosulphate (PMS; Sigma) for 3 hours. The amount of soluble formazan product formed by viable cells was quantified at 490 nm absorbance on a VersaMax microplate reader.

Samples	Sex	Immunophenotype	age at diagnosis (years)	Karyotype
TEL\AML1_1	F	pre-B-ALL	3	48,XX,add(19)(?p13),-?E,+21,+mar1,+mar2
TEL\AML1_2	M	pre-B-ALL	3	47,XY,t(12;2)(p1?3;p11),+21,inc[cp19]
TEL\AML1_3	F	C-ALL	5	46,XX,der(1)del(1)(p34)add(1)(q2?2),der(9)t(1;9)(q1?1;q1?2),der(9)t(1;9)(p3?4;q2),add(12)(p11~12),
TEL\AML1_4	F	C-ALL	4	46,XX[19]
TEL\AML1_5	M	pre-B-ALL	3	46,XY,5,der(5)(q?),?11,add(12)(p11),+mar[10][cp12]/46,XY[8]
TEL\AML1_6	F	C-ALL	4	cytogenetics failed; no data

Table 5: Primary sample information.

2.2.6 Flow cytometry

Cells were washed with wash buffer (PBS supplemented with 0.05% w/v sodium azide). Cells were pre-incubated with anti-mouse Fcγ III/II Receptor antibody (2.4G2; BD Biosciences) or human FcR-binding inhibitor (eBioscience) containing stain buffer (PBS supplemented with 0.05% w/v sodium azide and 1% w/v BSA) for 15 minutes on ice to block non-specific binding. The cells were then stained with fluorochrome-conjugated antibodies in stain buffer for 30 minutes on ice and washed with wash buffer prior to analysis. Anti-mouse or human antibodies used in this study are listed in Table 4. PE conjugated streptavidin (Ebioscience) was also used to detect biotin conjugated human CD2 antibody (Ebioscience).

For intracellular staining, human leukaemic cells were lysed and fixed in a single step using BD™ Phosflow Lyse/Fix buffer (BD Bioscience) at 37°C for the 10 minutes. The cells were then permeabilized with 500µl BD™ Phosflow Perm Buffer III (BD Bioscience) for 30 minutes on ice and stained with the appropriate antibody according to manufacturer's instructions (BD Biosciences). Appropriate IgG isotype control antibodies were used.

All flow cytometry was performed on an LRSII analyser (BD Biosciences) and the data was analysed with Summit 4.3 (Dakocytomation) or FlowJo v8.6 (Tree Star) software.

Name of antibodies	Supplier	Diluition
Mouse **TER119**-PE	BD Bioscience	1:200
Mouse **c-KIT**-APC (2B8)	Biolegend	1:200
Human **CD10**-PE (eBioCB-CALLA)	BD Bioscience	1:5
Human **CD19**-APC (HIB19)	human CD10-PE	1:5
Human **STAT3 Y705**-PE	BD Bioscience	20µl

Table 6: Flow cytometry antibodies used in this study.

PE: phycoerythrin; APC: Allophycocyanin

2.2.7 Apoptosis

Apoptosis was detected using the Annexin V Apoptosis Detection Kit I (eBioscience). Cells were washed with PBS followed by 1x Binding Buffer. The pellet was then resuspended in 95µl of 1x Binding Buffer and 5µl of Annexin V-APC or PE and incubated for 15 minutes at room temperature. The mixture was washed and resuspended in 300µl of 1x Binding Buffer and 5µl of 500µg/ml 4',6-diamidino-2-phenylindole (DAPI) or Propidium iodide (PI). Cells were then analysed by flow cytometry.

2.2.8 Cell cycle assays

Cell cycle analysis was performed using the Click-iT EdU Alexa Flour 647 Flow Cytometry Assay Kit (Invitrogen). Cells were cultured with 10µM Click-iT® EdU for 1.5 hour. The cells were then harvested and fixed with 100µl of Click-iT®

fixative for 15 minutes at room temperature. After one wash with PBS the cells were permeabilizated with 1X Click-iT® saponin-based buffer. Click-iT® reaction cocktail [Copper (II) sulphate ($CuSO_4$); 1:10 Reaction Buffer Additive (1M ascorbic acid); Fluorescent dye; TBS up to 500µl] was prepared and added to the cells. The reaction mixture was incubated for 30 minutes at room temperature, washed and resuspended in 500µL of 1X Click-iT® saponin-based permeabilization and wash reagent containing 2µg Ribonuclease A (Sigma) and 500µg/ml DAPI (Roche) or PI 1mg/ml. Cells were then analysed by flow cytometry.

2.2.9 Dead cell removal

Apoptosis and cell cycle analysis of shRNA transduced cells was performed 48 hours after treatment with the Dead Cell Removal kit and culture of equivalent numbers of viable cells (Miltenyi Biotec). Cells were centrifuged at 300g for 5 minutes, resuspended in 100µl of Dead Cell Removal MicroBeads and incubated for 15 minutes at room temperature. Column type MS was prepared by washing with 1x Binding Buffer and placed in a magnetic field of a MACS® Separator. 500µl of 1x Binding Buffer was added to the cells and the mixture was loaded into the column. The effluent was collected as the live cell fraction, centrifuged at 300g for 5 minutes and resuspended with the appropriate complete medium.

2.2.10 Proliferation assays

Cells were cultured at a density of $5x10^4$ cells per well in flat bottomed 96-well plates in the presence of dimethyl sulfoxide (DMSO) or specific inhibitors. DMSO was added at the same concentration as that present in the highest inhibitor concentration used. CellTiter 96® Aqueous One Solution Reagent (Promega, Madison, WI) was added to each well according to the manufacturer's instructions. After 96 hours in culture the cell viability was determined by measuring the absorbance at 490nm using a 550 BioRad plate-reader (BioRad) after 2 hours of incubation at 37 ºC.

2.2.11 Colony forming assays

For human cells, $1x10^4$ cells infected with shRNA vectors or treated with inhibitors were harvested, washed in PBS and resuspended in 600µl Cell Resuspension Solution (HSC002, R&D systems). The cells were added to 2.7ml of human methylcellulose (R&D system). 600µl of this mixture was plated into 24 multi-well plates using blunt end needles. The cells were cultured for 14 days before being cultured for an additional 2 days in the presence of 180µl of 10mg/ml 2-(P-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) in 75% ethanol, diluted 1:20 with PBS. The plate was then acquired using a calibrated densitometer (GS-800, BioRad) and the colonies quantified using ImageQuant TL software (GE Ealthcare).

2.3 Animals

All mice were maintained in the animal facilities of the UCL Institute of Child Health and experiments were performed according to United Kingdom Home Office regulations. Xenotransplantation in this study was performed by Dr. Owen William. 5-10 week old NOD.Cg-*Prkdc*^{scid}Il2rg^{tm1Wjl}/SzJ (NSG) mice were used as recipients for transplantation of scramble and shRNAs transduced Reh cells. These mice lack mature T cells, B cells, and natural killer (NK) cells. They are also deficient in multiple cytokine signalling pathways, and they have many defects in innate immunity. Specifically NSG mice associate the features of the NOD/ShiltJ background, the combined immune deficiency mutation (scid) and IL-2 receptor γ chain deficiency and have been shown to support greater engraftment of human haematopoietic stem cells (McDermott et al., 2010). Mice were sub-lethally irradiated with 3 Gy using a 137 Cs γ-irradiator and intravenously injected in the lateral tail vein with 1 x 10^5 transduced cells per mouse. Mice were sacrificed when they developed clinical signs of disease and bone marrow was harvested for analysis.

2.4 Statistical analysis

Statistical analysis of survival curves was performed using the Mantel-Cox log-rank test, and of SD by Student's *t* test.

CHAPTER III: Transcriptional TEL-AML1 regulated genes

3.1 Introduction

The use of microarray technology permits the simultaneous measurement of the expression of many thousands of genes, yielding large amounts of invaluable information and giving the opportunity to characterize many different biological processes. For example, in the cancer field, this technology is used to investigate the mechanisms by which a drug, disease, mutation and environmental conditions affect gene expression and cell function. In order to identify specific target genes whose expression is deregulated by TEL-AML1, in collaboration with Dr. Mike Hubank, who runs the ICH Gene Microarray Centre in our unit, our laboratory has used Affymetrix GeneChip MOE 430plus2.0 arrays to compare gene expression profiles from mouse haematopoietic progenitor cells (HPCs) expressing TEL-AML1 to those expressing an inactive TEL-AML1 point-mutant, that has been shown to impair DNA binding and TEL-AML1-induced self-renewal (Morrow et al., 2007; Roudaia et al., 2009). These results were further compared to cells transduced with empty retroviral vector. Using this approach, a number of genes that were specifically up- or down regulated in HPCs by TEL-AML1 were identified (Morrow et al., unpublished data).

In order to address the analysis of the transcriptional pathways involved in TEL-AML1 function, we first decided to confirm the changes in the levels of expression of the most interesting genes found to be up- or down-regulated in

the microarray data. Hence, using the same cDNA as that used for the microarray, we performed quantitative real-time PCR (qRT-PCR), a technique endowed with higher sensitivity and reproducibility than microarray analysis, using probes against the mRNA of the genes previously identified (Figure 3-1). Following this analysis we decided to further investigate three genes in the list: T-box transcription factor 2 (*TBX2)*, E2F transcription factor 5 *(E2f5)* and Leukaemia inhibitory factor receptor (*Lif-R*).

3.2 Results

3.2.1 TBX2 expression

TBX2 is a member of the T-box family of transcription factors and, with TBX3, is the only mammalian T-box factor that has been shown to function as a transcriptional repressor (Rowley et al., 2004). T-box proteins are DNA binding transcription factors that recognize similar sequences and their specificity in driving target gene induction or repression is hence only determined by association with different cofactors. These factors play a key role in cell identity maintenance during development. Moreover, recent work has suggested that they may play a relevant role in cancer. For example, TBX3 can cooperate with MYC and RAS in cellular transformation (Carlson et al., 2002), whereas TBX2 can suppress replicative senescence, regulating the promoter of *p21* and down regulating *p19 (Arf)* (Jacobs et al., 2000). In primary human breast cancer, *TBX2* has been found amplified in *BRCA1*- and *BRCA2*-

mutated breast tumours (Sinclair et al., 2002) whereas in other cancers, such as pancreas, liver and bladder, and in melanomas its expression has been found upregulated (Abrahams et al., 2010; Lu et al., 2010)

Here, we show a over-expression of the *Tbx2* gene in mouse HPCs transduced with TEL-AML1 retrovirus compared to cells transduced with empty vector or an inactive TEL-AML1 mutant, suggesting a direct role of the aberrant fusion protein in the regulation of *Tbx2* expression (Figure 3-2A).

In order to confirm the specificity of the over-expression of *TBX2* in presence of the t(12;21) translocation, we compared its expression in a panel of different human B-ALL with different genetic alterations (Table 4). The qRT-PCR results show that the transcript can be detected only in the cell lines in which TEL-AML1 is present, clearly demonstrating a relationship between the presence of TEL-AML1 and the over-expression of *TBX2* (Figure 3-2B).

3.2.2 E2F5 expression

The E2F proteins form a family of transcription factors that regulate the transition from the G1 to the S phase in the cell cycle, acting as modulators of the retinoblastoma tumour suppressor protein (pRb), or its related proteins p107 and p130 which are post transcriptionally regulated also by cyclin– cyclin-dependent kinase (CDKs) complexes (Chen et al., 2009). The E2F family has been traditionally divided into activator (E2F1–E2F3) and repressor (E2F4–E2F8) subclasses. Specifically, it has shown that the expression of *E2f4* and

E2f5 when associated with pocket proteins and other co-repressors are able to maintain the repression of E2F-responsive genes that promote entry into the G1 phase of the cell cycle (Gaubatz et al., 2000). However, analysis of E2F5 in other models suggests that it can be directly regulated by the cyclin E-Cdk2 complex and this interaction promotes cell cycle progression by recruitment of the p300/CBP family of co-activators (Morris et al., 2000). Additionally, it has been shown that E2F5, together with p107, acts as a transducer of transforming growth factor beta (TGFβ) receptor signals upstream of cyclin dependent kinases (Chen et al., 2002). Moreover, this factor may have a direct role in tumourigenesis since it has been found to be over-expressed in ovarian (De Meyer et al., 2009) and breast cancer (Polanowska et al., 2000), and the over-expression correlates with poorer clinical outcome (Umemura et al., 2009). This variability of E2F5 function can be explained by the fact that E2F family analysis is mostly based on the analysis of cells cultured *in vitro* but lacks *in vivo* validation (Chen et al., 2009).

Starting from our microarray dataset, showing an over expression of *E2f5*, we analysed its relative expression in a previously generated microarray database of cDNAs derived from patients with different B-ALL subtypes (Ross et al., 2003), the raw data being available in our lab. This analysis shows that the gene belongs to the list of genes differentially expressed in TEL-AML1 samples compared to the other types of leukaemia (data not shown). The expression data were further confirmed in a panel of different B-ALL cell lines by qRT-PCR (Figure 3-3A). However, the qRT-PCR data using HPCs

transduced with TEL-AML1 showed only a marginal over-expression of *E2f5* when compared to HPCs transduced with the TEL-AML1 mutant (Figure 3-3B).

3.2.3 Leukaemia inhibitory factor receptor

The leukaemia inhibitory factor receptor gene (*Lif-R*) encodes the cellular receptor for leukaemia inhibitor factor (LIF), a polyfunctional glycoprotein whose inducible production can occur in many tissues. This cytokine acts as a stimulus for platelet formation, proliferation of some haematopoietic cells, bone formation, adipocyte lipid transport, adrenocorticotropic hormone production, neuronal survival and formation, muscle satellite proliferation (Metcalf, 2003) and, mainly, in the maintenance of embryonic stem cells in an undifferentiated and pluripotent state (Williams et al., 1988). The partner receptor chain for LIF-R is gp130, a signalling subunit of the IL-6 cytokine receptor family that is responsible for the intracellular activation of the Janus-activated kinase-signal transducer and activator of transcription (JAK-STAT) and the mitogen-activated protein kinase (MAPK) signalling pathways (Bauer et al., 2007). Due to the presence of gp130, LIF-R is able to bind other cytokines, such as oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and cardiotrophin-1 (CT), and activate the same downstream signalling pathways (Metcalf, 2003). In our mouse model of TEL-AML1 over-expression, we found an increase in *Lif-R* mRNA expression (Figure 3-4). However, we were not able to confirm the expression of the

receptor using a conjugated antibody for flow cytometry in our TEL-AML1 human cell lines (data not shown).

3.3 Discussion

Identification of the molecular mechanisms controlling target gene selectivity and transcriptional control is essential to understand the function of TEL-AML1 in leukaemia. Here we show a specific over-expression of two genes involved in cell cycle, development and differentiation, and a gene involved in cytokine responses resulting from the presence of TEL-AML1, both in a mouse and human models.

Both TBX2 and E2F5 have been reported to regulate different phases of cell cycle progression, interacting with or directly inhibiting key factors involved in this process. Interestingly they act as regulators affecting the activity of the retinoblastoma protein (pRb) and its related proteins, p130 and p107 (Gaubatz et al., 2000; Vance et al., 2010), suggesting a possible role of this pathway in TEL-AML1 leukaemia. On the other hand, they also have important activities in several developmental processes, such us coordinating cell fate, patterning, and morphogenesis of a wide range of tissues and organs. Their transcriptional programs are poorly understood due to their involvement in such diverse processes and their context dependant activities, especially *in vitro*.

Due to its role as a polyfunctional cytokine receptor, LIF-R can affect many cellular mechanisms such as differentiation, survival, and proliferation. Mouse progenitor cells transduced with a TEL-AML1 retrovirus showed an increase in *Lif-R* gene expression but leukaemic TEL-AML1^{+} human cell lines did not show any LIF-R protein expression. Two different reasons could explain

this lack of expression by the human leukaemia cells. The first is that prolonged *in vitro* culture of the cells may have selected for intracellular mechanisms of signalling pathway activation, independent of cytokine signalling and therefore LIF-R expression. The second explanation may be connected to the different action of cytokines in the human and mouse models. Specifically, although LIF is able to activate the same signalling pathway in the two organisms, this has a different outcome in mouse and human embryonic stem cells failing to maintain the pluripotent state of human embryonic stem cells (hESCs) (Daheron et al., 2004). Additionally, we did not analyse whether TEL-AML1^{+} HPCs were able to induce the autocrine expression of LIF or whether they would respond to such production from the stromal niche. Further experiments are required in order to determine whether deregulation of *Lif-R* gene expression by TEL-AML1 in mouse HPC is an artefact of the mouse model, or has real significance for t(12;21) leukaemia. However, deregulation of this gene may also give insight into the wider signalling aberrations in TEL-AML1 leukaemia, something which is investigated in the next chapter.

In summary, since the main features of TEL-AML1 leukaemia are a block of B lymphocyte differentiation and uncontrolled proliferation, all of these factors may play an important role in the development, maintenance and progression of this disease

3.4 Figures

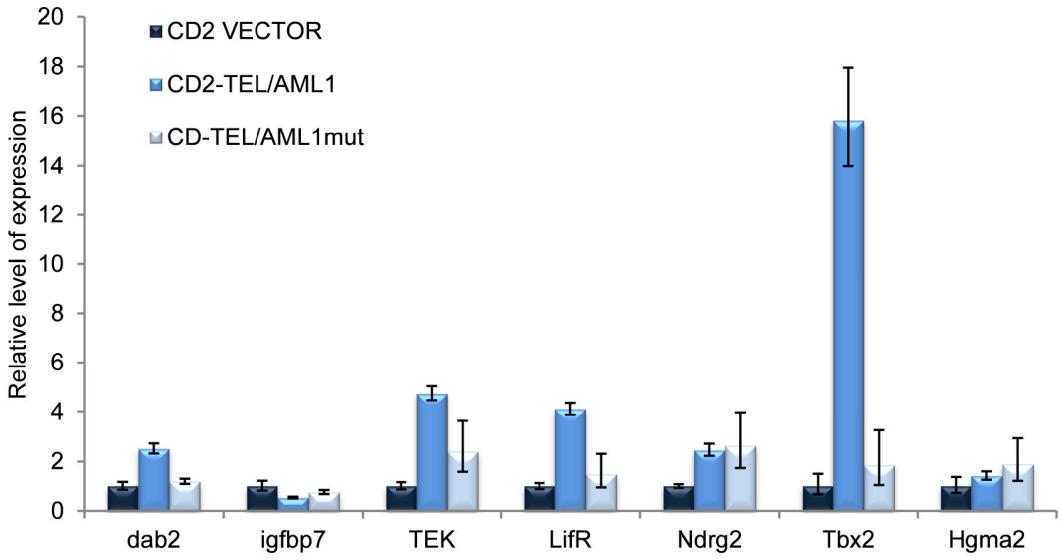


Figure 3-1: Confirmation of results obtained from microarray analysis.

Mouse foetal liver c-Kit^{+} Ter119^{-} cells were transduced with TEL-AML1 (blue bar), empty vector (dark blue bar) or a TEL-AML1 mutant (light blue bar) expressing retroviral vector. 72 hours later RNA was extracted and the level of relative gene expression of a selected list of genes was measured by qRT-PCR in order to confirm the results obtained from the previous microarray analysis. The bars show means ± s.d. of quadruplicate measurements. Data are representative of 2 independent experiments.

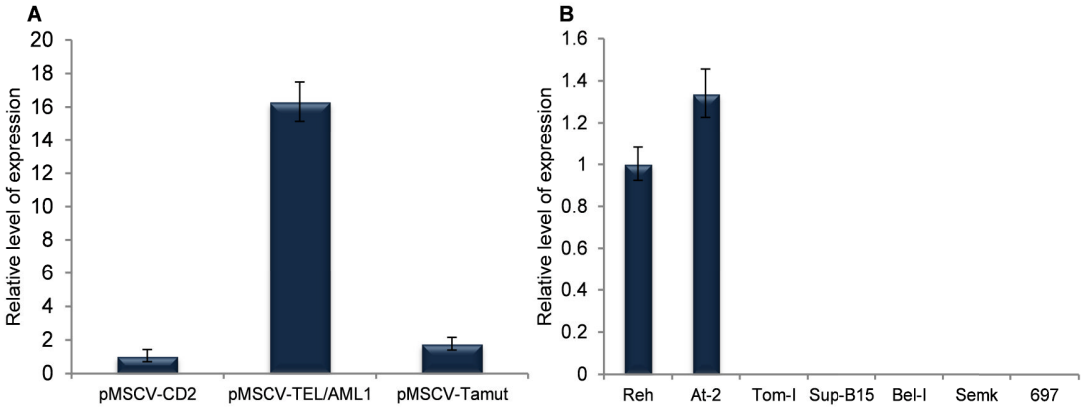


Figure 3-2: *Tbx2* expression analysis.

The bar charts represent the level of *Tbx2* expression in foetal liver c-Kit$^+$ Ter119$^-$ cells 72 hours after transduction with TEL-AML1 or a TEL-AML1 mutant (TAmut) constructs compared to the expression in cells transduced with the empty vector (**A**), and the comparison of *TBX2* expression in B-ALL human cell lines with different genetic alterations (**B**). All data are representative of three independent experiments and show means ± s.d. of quadruplicate measurements.

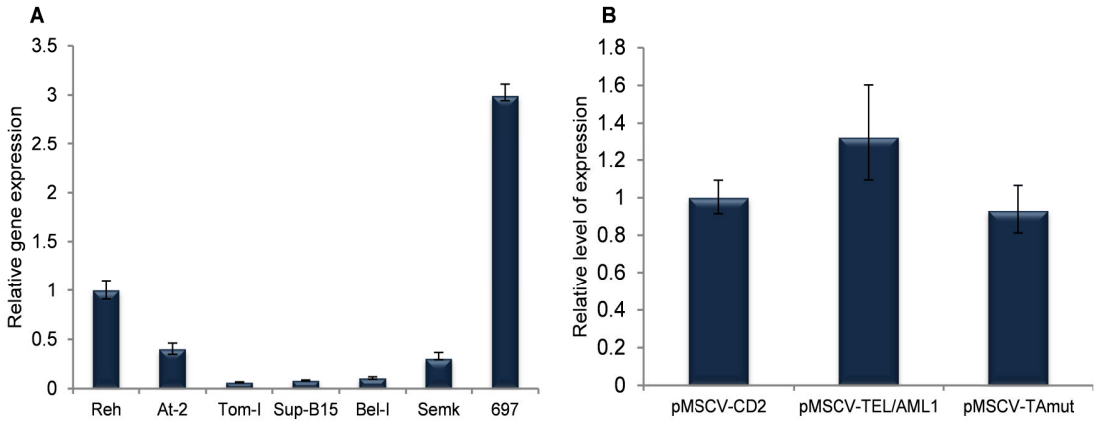


Figure 3-3: *E2f5* expression analysis.

The bar chart represents the comparison of *E2f5* expression in B-ALL human cell lines with different genetic alterations (**A**) and level of expression in foetal liver c-Kit^{+} Ter119^{-} cells 72 hours after transduction with TEL-AML1 or a TEL-AML1 mutant constructs compared to the expression of cell transduced with the empty vector (**B**). All data are representative of three independent experiments and show means ± s.d. of quadruplicate measurements.

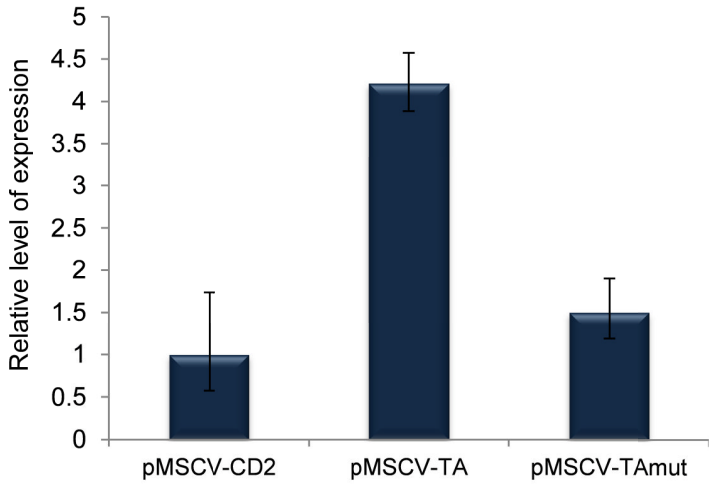


Figure 3-4: *Lif-R* expression analysis.

The bar chart represents the level of *Lif-R* expression in foetal liver c-Kit^{+} Ter119^{-} cells 72 hours after transduction with TEL-AML1 or a TEL-AML1 mutant constructs compared to the expression of cells transduced with the empty vector. All data are representative of two independent experiments and show means ± s.d. of quadruplicate measurements.

CHAPTER IV: Role of STAT3 in TEL-AML1 leukaemia

4.1 Introduction

One method of analyzing microarray data that is very informative is pathway analysis and many commercially available or free-software applications for pathway analyses on microarray data are now available. Using our dataset we decided to look at the signalling pathways affected following overexpression of the *TEL-AML1* fusion gene. Using Genespring software analysis and Ingenuity Pathway (IPA) software analysis we found that a subset of gene expression changes, such as altered *Lif-R* expression, highlighted activation of the signal transducer and activator of transcription 3 (STAT3) signalling pathway (Figure 4-1).

STAT3 belongs to of a family of seven transcription factors involved in different cellular biological processes. It was described for the first time as APRF (acute phase response factor), a DNA-binding factor activated by IL-6 and epidermal growth factor (EGF) able to induce acute-phase genes (Akira et al., 1994; Wegenka et al., 1993; Zhong et al., 1994). Subsequently, at least in cell culture systems, it was shown to be activated by diverse agents such as growth factors, oncogenes, and interferons (IFNs) (Levy and Lee, 2002).

All members of the STAT family have a very similar structure. Like all the other STAT proteins, STAT3 has an amino-terminal domain that is necessary for the dimerization of unphosphorylated STAT3 molecules, a DNA-binding domain, an SH2 domain involved in receptor recruitment and homo-

and hetero-dimerization, with another STAT3 molecule or other members of the STAT family (Caldenhoven et al., 1996), and a C-terminus transactivating domain (Figure 4-2A). An alternative isoform of STAT3, lacking part of the C-terminal domain (STAT3β) due to alternative mRNA splicing, has also been described. This isoform was originally believed to act as a competitive dominant negative. However, in more recent studies STAT3β has shown to be capable of regulating the expression of canonical STAT3 responsive genes as well as several other distinct target genes (Caldenhoven et al., 1996; Maritano et al., 2004; Schaefer et al., 1995; Yoo et al., 2002) (Figure 4-2B).

In order to be activated, STAT3 needs to be phosphorylated on the critical tyrosine (Tyr705) residue by receptor or intracellular tyrosine kinases. Phosphorylated molecules can then dimerize through reciprocal SH2 domain–Y705 interactions and translocate into the nucleus, where the complex is now able to bind to target sequences in specific promoters (Yu and Jove, 2004). However, different functions for dimerization have also been reported. Besides homo-dimerization, STAT3 can create hetero-dimers with distinct STAT proteins, causing different effects depending on the cell type. Generally hetero-dimers can act as sinks in order to reduce the pool of STAT proteins able to homodimerize, they can bind new specific sequences and they can modulate the transcriptional activity of STAT proteins by recruiting different co-activators or co-repressors. In addition, it has been shown that pre-existing complexes between non-phosphorylated STAT3 can be present in the cytoplasm, although a clear function for these has not been clarified (Schroder et al., 2004).

STAT3 contains a second phosphorylation site within its C-terminus. Classically the phosphorylation of this serine (Ser727) residue was considered to be a secondary event required for maximal transcriptional activity (Wen et al., 1995). However, it has been shown that regulation of STAT3 function by Ser727 phosphorylation can occur independently of Tyr705 phosphorylation, suggesting that this residue may have an independent role in the regulation of nuclear translocation and cell survival (Decker and Kovarik, 2000; Sakaguchi et al., 2012). Moreover, unphosphorylated STAT3 molecules can regulate the expression of genes distinct to those regulated by phosphoprylated STAT3 dimers. Although these studies are preliminary and further work is required to elucidate the different mechanisms regulating the transcription factor activity of STAT3, they illustrate the importance of this transcription factor in regulating many cellular functions. However, in addition to regulating transcription, recent evidence has also suggested important non-transcriptional functions of STAT3. For example, STAT3 has been shown to stabilize the polymerization of microtubules by directly binding stathmin (Ng et al., 2006), a small tubulin-binding protein. More importantly, STAT3 has also been shown to be involved in cellular respiration in the mitochondria by regulating the activities of complexes I and II of the electron transport chain (Wegrzyn et al., 2009).

4.1.2 STAT3 in cancer and leukaemia

STAT3 can be activated in a wide variety of signalling pathways, and for this reason it is able to regulate many fundamental processes necessary for the

survival and proliferation of cells. This is evident in light of the fact that disruption of the *STAT3* gene in mice leads to early embryonic lethality (Takeda et al., 1997). For this reason it is not surprising that STAT3 plays an important role in oncogenesis and leukaemogenesis.

Aberrant overexpression and/or persistent activation of the protein have been reported in the majority of human solid and haematological tumours (Yu and Jove, 2004) (Table 7). Initial studies demonstrated that persistent activation of STAT3 in immortalized fibroblasts causes cellular transformation and tumour formation in nude mice (Bromberg et al., 1999). Subsequently, STAT3 function was also associated with oncogenic transformation mediated by various different oncoproteins, such as sarcoma viral oncogene homolog tyrosine kinase (v-SRC), breakpoint cluster region (BCR) - Abelson, epidermal growth factor receptor (EGFR) and rat sarcoma (RAS) (Bromberg et al., 1998; Frank and Varticovski, 1996; Gough et al., 2009; Grandis et al., 1998). In fact, constitutive activation of STAT3 affects the function of many factors commonly deregulated in human cancer. For example, STAT3 has shown to bind the promoter of the *TP53* gene and inhibit its expression (Niu et al., 2005), to regulate the expression of thymoma viral oncogene homolog (AKT) (Xu et al., 2005) and to prolong NF-κB nuclear retention (Lee et al., 2009). Recent evidence also suggests a possible role for somatic *STAT3* mutations in cancer. Thus, activating *STAT3* mutations have been discovered in 40% of T-cell large granular lymphocytic leukaemia (Koskela et al., 2012).

In conclusion, STAT3 is considered to play a fundamental role in carcinogenesis and tumour development.

4.1.3 STAT3 inhibition

The inhibition of aberrant STAT3 activation by genetic approaches has been shown to induce growth arrest and apoptosis of a wide range of transformed cells *in vitro (Chiarle et al., 2005; Konnikova et al., 2003; Li et al., 2009).* For this reason, STAT3 has emerged as an attractive target to inhibit both oncogenesis and leukaemogenesis using pharmacological methods (Figure 4-3). One strategy to obtain such inhibition is using an indirect method to target the upstream regulators of STAT3. To this category belong agents capable of inhibiting receptors involved in activation of STAT3 signalling, such as the anti-CD20 antibody (Rituximab), that has been tested in non-Hodgkin's lymphomas (Alas and Bonavida, 2001), and the EGFR-directed antibodies (Hynes and Lane, 2005). A alternative approach to blocking upstream regulators of STAT3 is using specific tyrosine kinase inhibitors able to disrupt STAT3 activation, such as JAK kinase and SRC kinase inhibitors (Ferrajoli et al., 2007; Iwamaru et al., 2007; Pardanani et al., 2007; Warmuth et al., 2003). However, the most specific and effective method of inhibiting the aberrant activity of STAT3 is directly targeting one of the three functional domains (SH2 domain, DNA binding domain and N-terminal domain) of the protein in order to block its activity (Figure 4-3). These specific inhibitors can be further classified

into two different categories: (i) peptide and peptidomimetics; (ii) small molecules nonpeptidic inhibitors.

The largest class of inhibitors that target STAT3 directly is represented by inhibitors that interact with the SH2 domain, preventing the binding of the STAT3 SHR domain to phosphotyrosine motifs in receptors and the homodimerization of STAT3 molecules. These inhibitors block the activation of STAT3 and silence STAT3-mediated gene transcription, causing cell growth inhibition and apoptosis in different cancer models. On the other hand, a few studies have also shown the efficacy of blocking cancer cell proliferation using DNA-binding and N-terminal inhibitors of STAT3. Some platinum compounds, for instance, have been reported to potentially block the DNA binding activity of STAT3, whereas a category of short peptides that recognize the N-terminal domain of STAT3 were also capable of inhibiting its activity [reviewed in (Debnath et al., 2012; Page et al., 2011; Yue and Turkson, 2009)].

Although many STAT3 inhibitors have been developed, only a small number have been used in clinical trials. Three of these, Pyrimethamine, OPB-31121 and RTA 402, are currently being tested in clinical trials of haematological cancers (http://clinicaltrials.gov). However, the data from these trials are not publically available at present.

4.2 Results

4.2.1 Inhibition of STAT3 induces apoptosis and a cell cycle block

In leukaemia, activation of STAT3 has been shown to play a role mainly in Acute Myeloid Leukaemia (AML) whereas alteration of STAT5 is more frequent in Acute Lymphoblastic Leukaemia (ALL) (Benekli et al., 2009). Based on our microarray pathway analysis we decided to test the role of these two factors in the context of TEL-AML1$^+$ leukaemia, measuring the proliferation and survival of human leukaemic cell lines in the presence of specific inhibitors. We analysed two different cell lines carrying the t(12;21) translocation (Reh, At-2), two with BCR-ABL rearrangement, representing a known STAT3 dependent oncogenic rearrangement (Coppo et al., 2003) (Tom-I, Sup-B15), and three with different genetic alterations (697, Bel-I, Semk) (Table 4). The TEL-AML1$^+$ leukemic cell lines demonstrated a greater sensitivity to treatment with the specific STAT3 inhibitors S3I-201, Stattic and Inhibitor VII, than the non TEL-AML1$^+$ leukemic cell lines, greater even than that of the BCR-ABL cells (Figure 4-4A, B and C). These inhibitors have been shown to be able to selectively inhibit activation, dimerization, and nuclear translocation of STAT3 (Schust et al., 2006; Siddiquee et al., 2007). In contrast, although activation of STAT5 has previously been associated with t(12;21) leukaemia, as a consequence of EpoR up-regulation (Torrano et al., 2011), its pharmacological inhibition did not block the proliferation of Reh and At-2 cell lines (Figure 4-5).

In order to determine whether the sensitivity of the TEL-AML1$^+$ cell lines to STAT3 inhibition was due to induction of cell death or a cell cycle block, we

evaluated the level of apoptosis and the effect on the cell cycle profile in cells exposed to 50μM S3I-201 for 24 hours. Treatment of cells with this inhibitor resulted in a substantial increase of apoptosis (Figure 4-6) and an almost complete block of the cell cycle, in the G_1-S phase transition (Figure 4-7). In order to confirm these data molecularly, we analysed the level of cleaved caspase 3, as a marker of apoptosis, and the expression of p21 and p27 proteins, as markers of cell cycle progression. Increased expression of both p21 and p27 (Figure 4-8A) was readily detected after drug treatment, which also resulted in augmented Cleaved caspase 3 (Figure 4-8B), confirming that the S31-201 inhibitor induces both cell death and cell cycle arrest.

In order to further confirm the sensitivity of the TEL-AML$^+$ cell lines to STAT3 inhibition, we decided to silence the gene in these cells using small hairpin RNA (shRNA) constructs. A set of 5 different shRNA targeting STAT3 was tested, from which we identified two independent hairpins capable of reducing the expression of STAT3 by approximately 90% (Figure 4-9A and B). STAT3 silencing resulted in induction of apoptosis and cell cycle inhibition, similar to what we saw with the inhibitor (Figure 4-9C and D). Taken together, these data indicate that STAT3 activity is of fundamental importance for the proliferation and survival of human TEL-AML1$^+$ cell lines.

4.2.3 TEL-AML1 is able to regulate the level of STAT3 phosphorylation

Our lab has previously established a method to study the effect of over-expressing the TEL-AML1 fusion protein in mouse foetal liver haematopoietic progenitor cells (HPC). In brief, c-kit^{+} Ter119^{-} HPC were purified from embryonic day 13 (E13) mouse foetal livers and transduced with a retroviral vector expressing TEL-AML1 (see material and methods). The overexpression of the fusion gene in these cells was previously shown to promote B-lymphocyte development, enhance self-renewal of B-cell precursors, and lead to the establishment of long-term growth factor-dependent pre-B-cell lines (Morrow et al., 2004). In order to examine the putative connection between TEL-AML1 and STAT3, we decided to analyse the effect of TEL-AML1 expression on STAT3 activation in HPCs. Transduction of HPCs with TEL-AML1 expressing retrovirus resulted in increased levels of STAT3 phosphorylation at both Y705 and S727 residues (Figure 4-10A, B and C).

To determine whether STAT3 activation was still dependent upon TEL-AML1 expression in the human leukaemia cell lines, we decided to knock-down TEL-AML1 expression in Reh cells. Because the second, non-mutated allele of *TEL* is lost in Reh cells (Figure 4-11A), we were able to specifically target TEL-AML1 using a shRNA directed against the 5' part of *TEL*. This shRNA resulted in silencing of *TEL-AML1* RNA, as detected by qPCR (Figure 4-11B). In order to confirm the efficacy of *TEL-AML1* silencing, we also decided to measure changes in fusion protein expression by Western blot analysis. Based on

previously published data, we initially tested three different antibodies; two recognizing the N-terminus of TEL and one the Runt Homology Domain (RHD) of AML1 (Diakos et al., 2010; Gefen et al., 2010; Zaliova et al., 2011). All these antibodies were tested using both nuclear and cytoplasmic extracts of human leukaemic cells. Although all of these antibodies recognized a band at the correct molecular weight for the TEL-AML1 protein, the band recognized appeared to be non-specific, since it was also present in the lanes containing extracts from non-TEL-AML1 leukaemic cells (Figure 4-12). The non-specific band was still present after changing the percentage of the gels, time of antibody incubation and the washing conditions. The specific TEL-AML1 band was finally identified in nuclear extracts using a polyclonal antibody directed against the helix-loop-helix (HLH) domain of TEL (king gift of Jan Cools, Belgium). Using this antibody we confirmed that the *TEL*-specific shRNA resulted in knock-down of TEL-AML1 protein in Reh cells (Figure 4-13).

Two different studies have previously investigated the effect of *TEL-AML1* silencing in human leukaemic cell lines. *Zaliova et al* in 2011 used siRNA transfection to target TEL-AML1. They reported that TEL-AML1 depletion did not alter the survival, proliferation and clonogenic potential of Reh cells, and indeed resulted in no significant changes in gene expression in the transfected cells (Zaliova et al., 2011). In contrast, *Fuka et al* in 2012 used stable shRNA expression to demonstrate that *TEL-AML1* knock-down resulted in cell cycle inhibition and impaired engraftment of Reh cells in a xenotransplantation mouse model (Fuka et al., 2012). Our data are consistent with the latter study,

knock-down of TEL-AML1 with our construct resulting in a G1-S phase arrest (Figure 4-14A) and a reduction in clonogenic activity of Reh cells in a methylcellulose based colony-forming unit assay (CFU assay) (Figure 4-14B). In order to consolidate the importance of the fusion gene for the survival and proliferation of the leukemic cells, we transduced the Reh cell line with a TEL-AML1 mutant. This mutant lacks of the the runt homology domain (RHD) and it has been shown previously to abolish TEL-AML1 binding to RUNX1 DNA consensus sequences (Morrow et al., 2007). Our hypothesis was that overexpression of this mutant could act as a dominant negative mutant with respect to TEL-AML1, binding the fusion protein through protein-protein interactions between the respective TEL moieties. Although this hypothesis still requires careful validation, overexpression of the mutant protein resulted in increased apoptosis, a block in the cell cycle and reduced colony formation in methylcellulose. These data are entirely consistent with the results from shRNA mediated silencing of the fusion gene and highlight the importance of TEL-AML1 for the survival of leukaemic clones (Figure 4-15A-C).

Furthermore, and confirming the link between the fusion protein and STAT3 activity observed in mouse HPC, TEL-AML1 silencing in Reh cells resulted in reduced levels of phosphorylated Y705-STAT3 (Figure 4-16).

4.2.4 Activation of STAT3 is necessary for the self-renewal and leukaemogenic activity of TEL-AML1

By definition leukaemia is a malignant disease of the blood-forming organs, characterised by abnormal proliferation and development of leukocytes and their precursors in the blood and bone marrow. In normal conditions, proliferation and self-renewal of precursor cells is a process highly regulated by networks of proto-oncogenes and tumour suppressors. In contrast, in the malignant context self-renewal appears to be one of the main deregulated processes, as a consequence of severe imbalances within such networks.

STAT3 has been shown to be important for self-renewal activity in different types of cancer, such as glioblastoma and breast cancer (Dave et al., 2012; Guryanova et al., 2011). For this reason, we decided to analyse the clonogenic capacity of TEL-AML1$^+$ cell lines in the presence of pharmacological or molecular inhibition of STAT3. The ability of TEL-AML1 expressing cells to form colonies in CFU assays was specifically inhibited by the addition of the drug S3I-201 when compared to non-TEL-AML1$^+$ cells (Figure 4-17), or by silencing *STAT3* expression (Figure 4-18), suggesting that blocking STAT3 activity is not only responsible for a decrease in viability of the bulk of the cell lines, but it also inhibits their self-renewal capacity.

In order to address the importance of STAT3 in leukaemia progression *in vivo*, we next transplanted immunocompromised NSG mice with Reh cells following *STAT3* silencing (Figure 4-19). Although all of the transplanted mice developed leukaemia, the latency of leukaemia onset was significantly greater

in those transplanted with *STAT3*-specific shRNA transduced cells (Figure 4-20A). Moreover, increased levels of *STAT3* mRNA expression were detected in the shRNA transduced cells recovered from leukaemic mice, in comparison to those observed in the cells pre-transplantation (Figure 4-20B). This demonstrates *in vivo* selection against leukemic cells with low *STAT3* expression. Similar selection against cells expressing low levels of *STAT3* was also seen in long-term *in vitro* cultures of transduced Reh cells (Figure 4-20C and D).

4.2.5 Human TEL-AML1$^+$ primary leukaemia cells are sensitive to S3I-201

In order to analyse the importance of STAT3 in primary leukaemia samples, we collaborated with Professor Monique den Boer's group at the Erasmus MC institute in Rotterdam. They tested the sensitivity of four different TEL-AML1$^+$ primary leukaemia samples (Table 5) to different concentrations of S3I-201. The dose response curves obtained show very similar sensitivity of the primary leukaemia cells to that displayed by the Reh cell line (Figure 4-21A). We obtained frozen vials of two further TEL-AML1$^+$ primary leukaemia samples, in order to examine their responses to STAT3 inhibition in more detail. Although these leukaemia cells exhibited relatively high levels of spontaneous apoptosis following *in vitro* culture, treatment of the cells with S3I-201 during this culture period caused a significant increase in apoptosis (Figure

4-21B). This data indicates that TEL-AML1^{+} primary leukaemia cells are also highly dependent on STAT3 activity.

4.3 Discussion

The molecular pathways regulated by TEL-AML1 remain largely unknown. Here we present evidence for the novel role of STAT3 signalling in the maintenance of TEL-AML1 driven leukaemia.

Mouse progenitor cells transduced with a TEL-AML1 expressing retrovirus showed an increase in phosphorylation of STAT3 whereas knock-down of the fusion gene in human TEL-AML1$^+$ leukemic cell lines resulted in reduced levels of phospho-STAT3. Taken together, these results demonstrate a role for TEL-AML1 in regulating the activity of STAT3. Moreover, human leukaemic cell lines carrying this translocation exhibited increased sensitivity to STAT3 inhibition, especially with S3I-201, in comparison to leukaemic cells containing distinct genetic aberrations. STAT3 inhibition with S3I-201 and two independent shRNA constructs, resulted in apoptosis, cell cycle arrest and impairment of the clonogenic activity of TEL-AML1$^+$ cells. Furthermore our data indicate that STAT3 is important for the progression of leukaemia in an *in vivo* experimental model and, more importantly, for the survival of primary patient leukaemia cells.

Although STAT5 rather than STAT3 has been previously associated with ALL, treatment of TEL-AML1$^+$ human cell lines with a STAT5 inhibitor did not affect their proliferation. This suggests that at least *in vitro* this factor is of minor importance in TEL-AML1 leukaemia. STAT3 function on the other hand has been implicated in AML rather than ALL pathogenesis and despite it being a

well-studied transcription factor, little is known about its function during the B-lymphocyte development. Recent work has suggested a possible role for STAT3 in early B cell development, probably acting at the pre-pro B transition by contributing to the survival of IL-7 responsive progenitors (Chou et al., 2006). Thus, IL-7 has been shown to increase the activity of STAT1, STAT5 and STAT3 in B progenitor cells, thereby regulating the survival and the proliferation of these cells (van der Plas et al., 1996) but not of mature B cells (Henney, 1989). Furthermore, IL-7 receptor (IL-7R) gain-of-function mutations, causing constitutive activation of STAT and mTOR pathways, have been reported in paediatric B-ALL (Shochat et al., 2011). However, although IL-7R signalling may explain STAT3 activation in the B cell progenitor pool susceptible to transformation by TEL-AML1, a study demonstrating suppression of IL-7R expression upon expression of this fusion gene in mouse bone marrow cells suggests that this pathway is unlikely to play an important role in STAT3 activation in TEL-AML1$^+$ leukaemia (Tsuzuki et al., 2004).

Similar to the results obtained with TEL-AML1$^+$ cell lines, we also demonstrated a high sensitivity of BCR-ABL$^+$ ALL cell lines to STAT3 inhibition. In ALL, this fusion has been associated with constitutive activation of STAT5, even though, there is evidence that BCR-ABL is able to activate other members of the STAT family (Ilaria and Van Etten, 1996) in distinct haematopoietic diseases, such as chronic myeloid leukaemia (CML) (Coppo et al., 2003). Our data suggest that activation of STAT3 is not exclusive to TEL-

AML1^{+} ALL and that STAT3 may be a therapeutic target in different subtypes of ALL, as well as in AML.

ETV6 has been shown to repress the transcriptional activity of STAT3 (Schick et al., 2004) whereas, in contrast, RUNX1 has a synergistic effect with STAT3 in gene transcription activation (Yanagida et al., 2005). The genes encoding these transcription factors, besides being involved in the TEL-AML1 translocation itself, also represent two of the most frequent targets of secondary alterations in TEL-AML1^{+} leukaemia. Specifically, *ETV6* is lost in more than 60% of the cases (Kempski and Sturt, 2000) whereas increases in *RUNX1* copy number occur in almost 25% of the cases (Chung et al., 2010). For this reason secondary mutations, such as loss of the wild-type *ETV6* allele and acquisition of additional *RUNX1* copies, may be progressive due their effects on the transcriptional regulation of STAT3 target genes.

It is unclear how TEL-AML1 deregulates STAT3 activation. One possible way in which this could be achieved would be via interaction between the fusion protein and STAT3 in the nucleus, through direct binding or via co-factors such as Sin3a, which has been shown to bind both TEL-AML1 and STAT3 (Icardi et al., 2012). Another possibility is transcriptional deregulation by TEL-AMI1 of intracellular factors able to activate STAT3 or, *in vivo,* of particular cytokines or growth factors capable of inducing STAT3 signalling.

In conclusion we showed for the first time an aberrant STAT3 activation that is necessary for the survival, proliferation and self-renewal of TEL-AML1^{+} ALL.

4.4 Figures

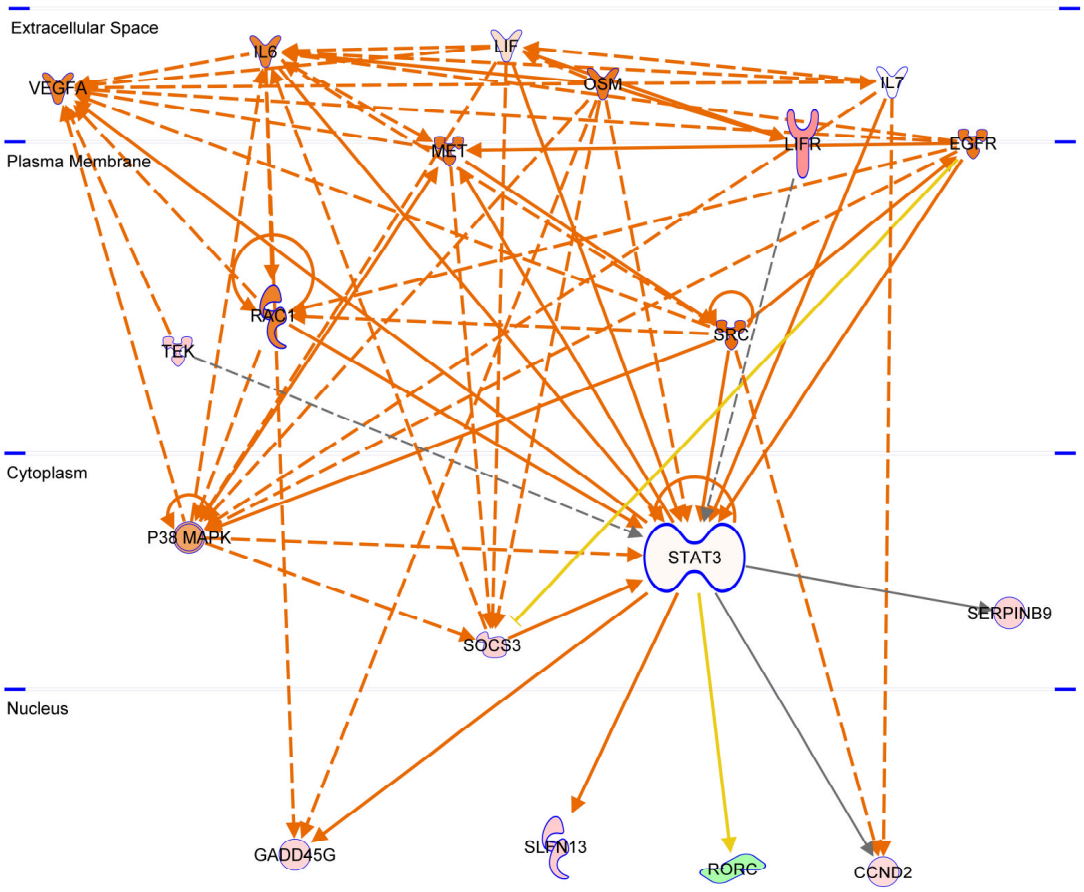


Figure 4-1: Network analysis of TEL-AML1 target gene expression.

The diagram shows the analysis of networks of genes down- or up-regulated in HPCs transduced with a TEL-AML1 retroviral vector, after normalization to TEL-AML1 mutant and control empty vector (see section 3.1). The analysis revealed that one of the main common factors altered, after combining all the pathways, was STAT3. The figure indicates that some targets of TEL-AML1, shown in red (up-regulated) and green (down-regulated), may also be directly regulated by STAT3. In order to facilitate comprehension of the figure all the

genes up- or down-regulated that are responsible, upstream or downstream, of the regulation of the pathways indicated and are not directly linked to STAT3 have been removed.

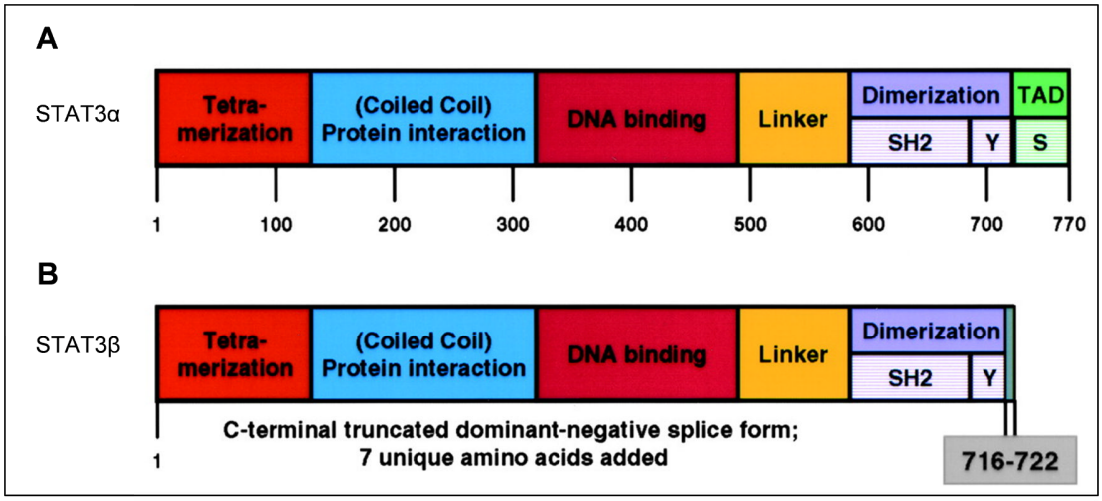


Figure 4-2: Schematic structure of STAT3 isoforms [Adapted from (Buettner et al., 2002)].

The diagrams show the full length STAT3 protein (STAT3α) and the STAT3 splice variant (STAT3β). The two isoforms are characterized by the presence of an N-terminus domain that mediates the STAT dimer-dimer interaction to form a tetramer, a coiled coil for protein interaction and a DNA binding domain. The phosphorylation of a tyrosine residue in the dimerization region mediates the interaction with the SH2 domain of another monomer that stabilizes STAT dimer formation and activates its high affinity DNA binding. The C-terminus domain is a transcriptional activation domain (TAD) regulated by serine phosphorylation. The STAT3β splice variant contains a COOH- terminal deletion resulting in a loss of the TA domain.

Tumour type	Activated STAT
Blood tumours	
Multiple myeloma	STAT1, STAT3
Leukaemias:	
HTLV-I-dependent	STAT3, STAT5
Erythroleukaemia	STAT1, STAT5
Acute myelogenous leukaemia (AML)	STAT1, STAT3, STAT5
Chronic myelogenous leukaemia (CML)	STAT5
Large granular lymphocyte leukaemia (LGL)	STAT3
Lymphomas:	
EBV-related/Burkitt's	STAT3
Mycosis fungoides	STAT3
Cutaneous T-cell lymphoma	STAT3
Non-Hodgkins lymphoma (NHL)	STAT3
Anaplastic large-cell lymphoma (ALCL)	STAT3
Solid tumours	
Breast cancer	STAT1, STAT3, STAT5
Head and neck cancer	STAT1, STAT3, STAT5
Melanoma	STAT3
Ovarian cancer	STAT3
Lung cancer	STAT3
Pancreatic cancer	STAT3
Prostate cancer	STAT3

Table 7: Abnormal activation of STAT proteins in human cancers [Adapted from (Yu and Jove, 2004)].

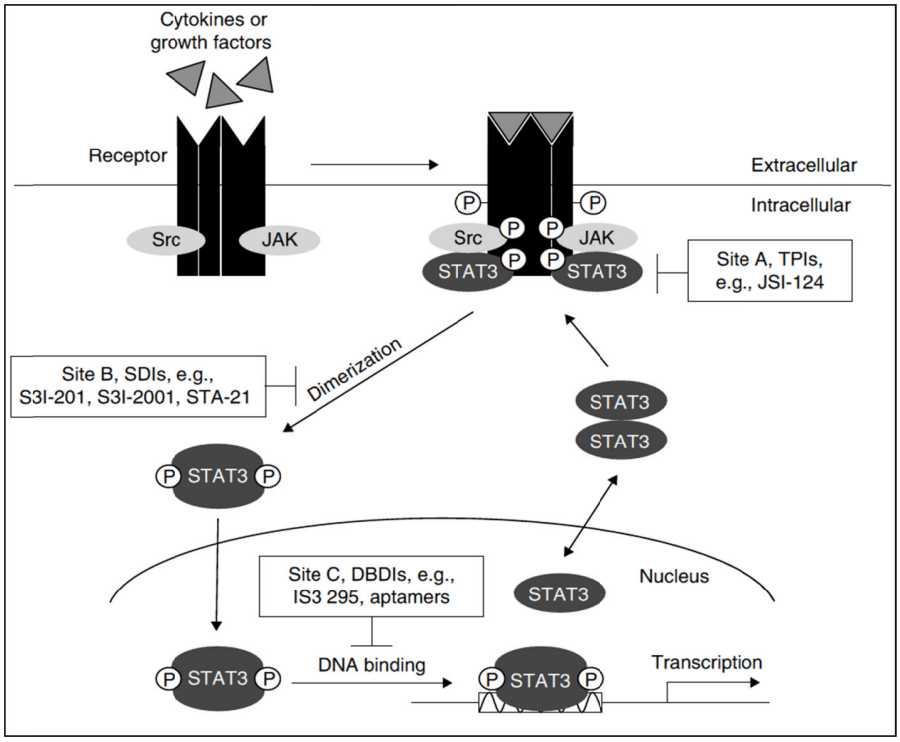


Figure 4-3: Specific targeting site in the STAT3 signalling pathway [Adapted from (Yue and Turkson, 2009)].

The figure shows the possible sites of targeting the STAT3 signalling pathway and examples of available inhibitors for each one. The first strategy is to inhibit the binding to their cognate receptors (site A). Alternatively small molecules have been designed to prevent the dimerization STAT3:STAT3 that allow the molecules to be fully activated and to migrate into the nucleus (site B). The most challenging strategy is to interfere with the DNA binding ability of STAT3 (site C).

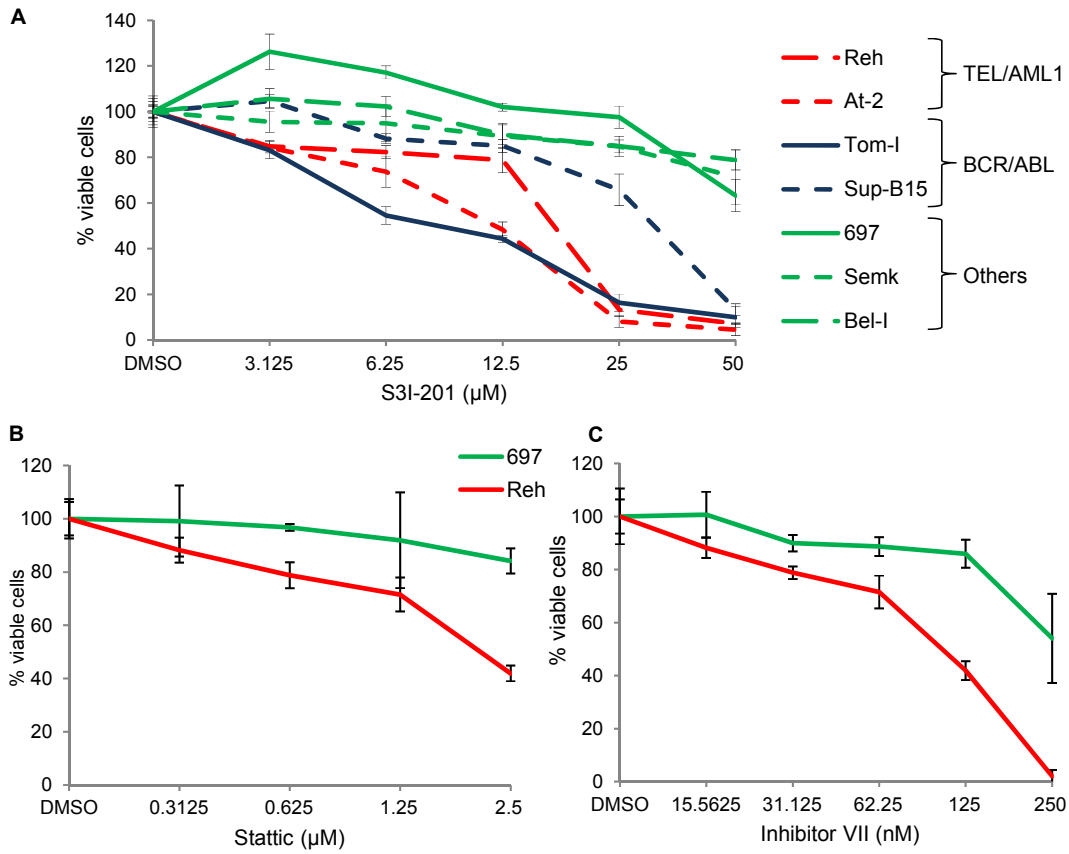


Figure 4-4: Pharmacological inhibition of STAT3 leads to a specific block of proliferation in human TEL-AML1$^+$ cell lines.

The chart shows the proliferation of leukaemic cell lines as measured by MTS assay after 96 hours culture with the indicated concentrations of the following STAT3 inhibitors: S3I-301 (**A**), Stattic (**B**) and Inhibitor VII (**C**). Results are normalized to the proliferation of DMSO treated cells. All data are representative of three independent experiments and show means ± s.d. of triplicate measurements.

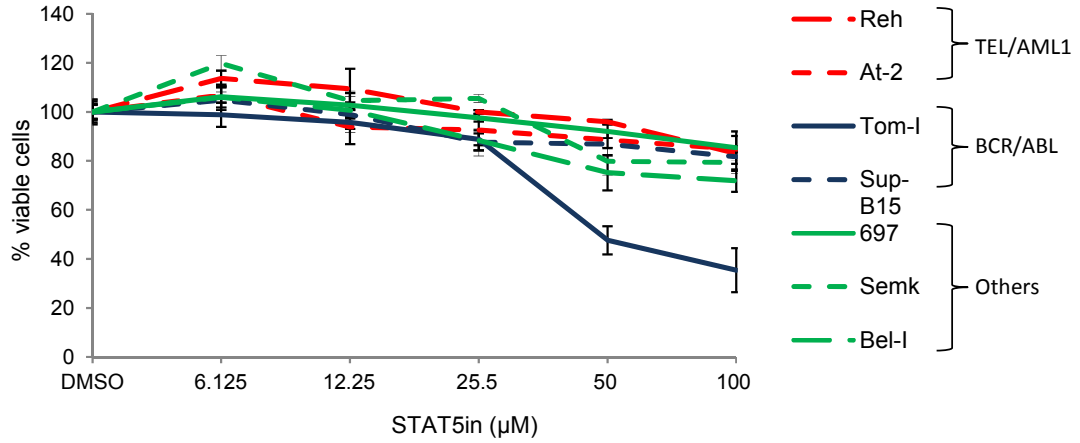


Figure 4-5: STAT5 inhibition does not affect TEL-AML1+ cell line proliferation.

The chart shows the proliferation of leukemic cell lines as measured by MTS assay after 96 hours culture with the indicated concentrations of STAT5 inhibitor (STAT5in). Results are normalized to the proliferation of DMSO treated cells. All data are representative of three independent experiments and show means ± s.d. of triplicate measurements.

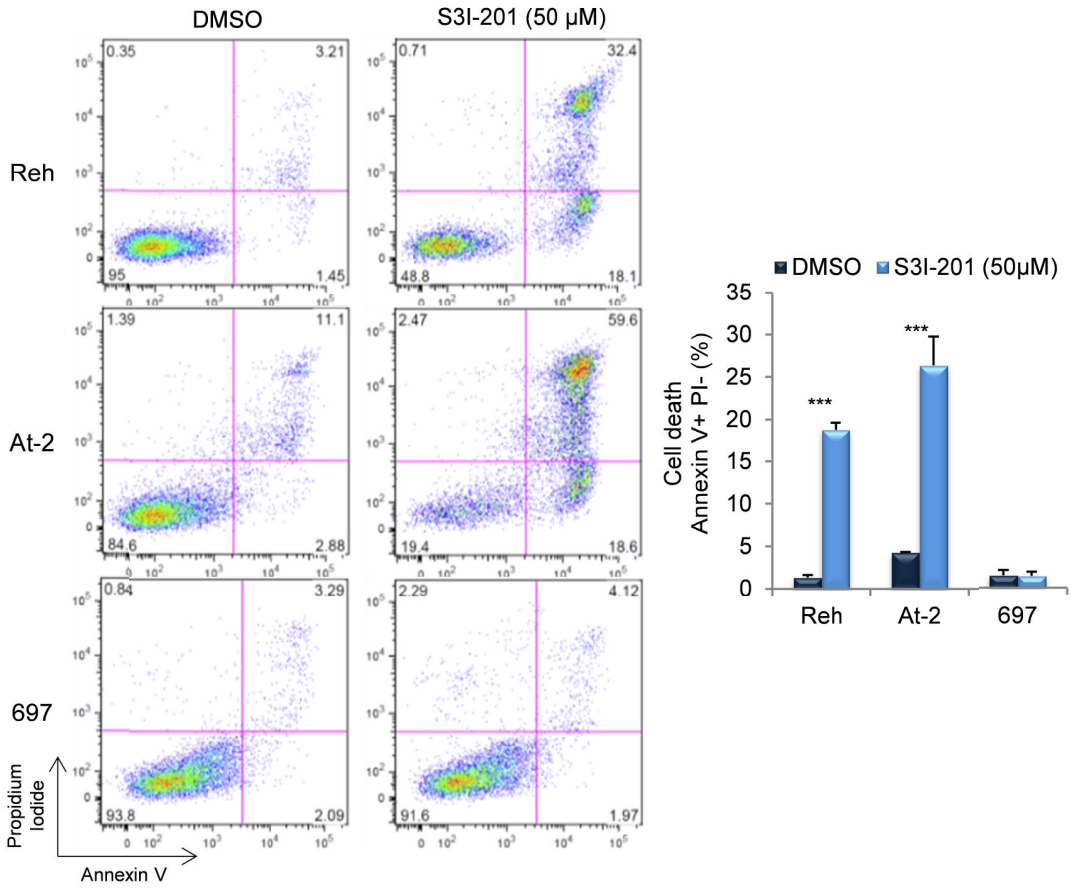


Figure 4-6: S3I-201 treatment causes apoptosis in TEL-AML1^{+} cell lines.

The FACS plots are examples of the apoptosis profile obtained in 3 independent experiments. Apoptosis was evaluated after treating the cells with DMSO or 50µM S3I-201 and staining with Annexin V at 24 h. The bar chart represents the quantification of the percentage of apoptotic Annexin V^{+}PI^{-} cells after drug treatment normalized to the percentage of DMSO treated cells. All data are representative of three independent experiments and show means ± s.d. of triplicate measurements. *P < 0.05, **P < 0.01, ***P < 0.005 compared to control (Student's unpaired t test).

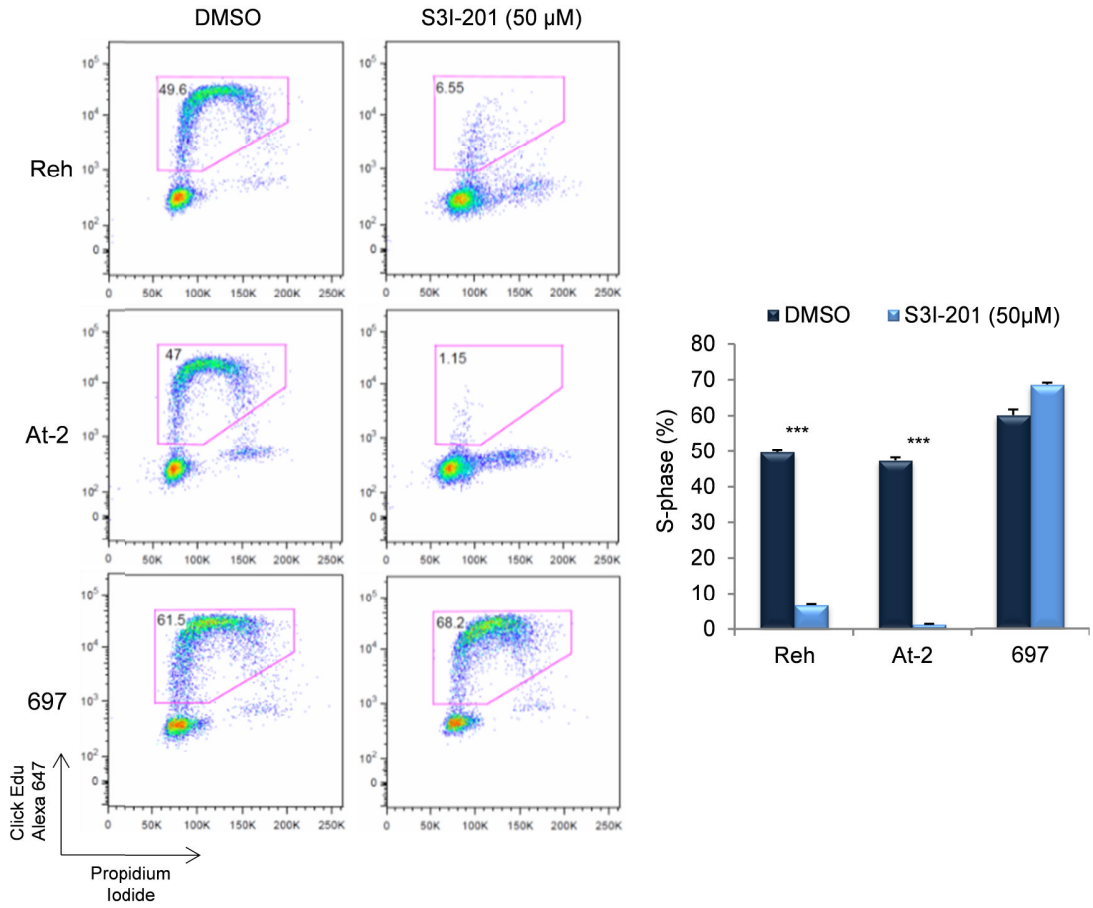


Figure 4-7: S3I-201 treatment causes cell cycle block in TEL-AML1^{+} cell lines.

The FACS plots are examples of the cell cycle profile obtained in 3 independent experiments. Cell cycle profile was evaluated after treating the cells with DMSO or 50µM S3I-201 for 24h and pulsing with 10µM 5-ethynyl-2´-deoxyuridine (Edu) for 1 hour. The bar chart represents the quantification of the percentage of S-phase cells after drug treatment normalized to the percentage of DMSO treated cells. All data are representative of three independent experiments and show means ± s.d. of triplicate measurements. *P < 0.05, **P < 0.01, ***P < 0.005 compared to control (Student's unpaired t test).

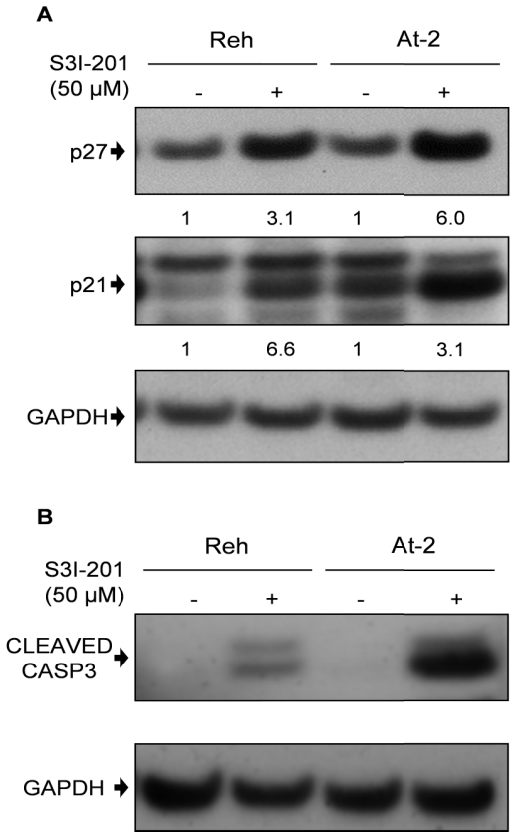


Figure 4-8: S3I-201 increases p21 and p27 protein expression and caspase 3 cleavage.

The figure shows the western blot analysis of p21, p27 (**A**) and cleaved caspase 3 (**B**), in Reh and At-2 cells 24 hours after treatment with 50μM S3I-201 or DMSO. Numbers represent densitometric quantitation of p21 and p27 bands normalized to GAPDH (**A**). Data are representative of 2 (**A**, p21) and 3 (**A**, p27 and **B**) independent experiments.

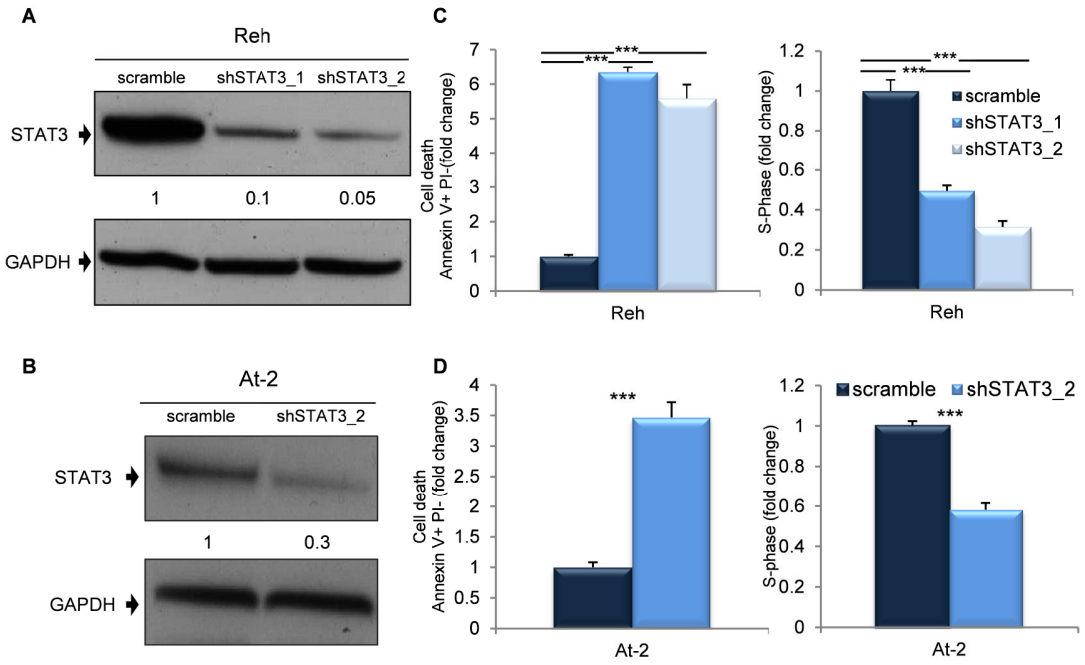


Figure 4-9: *STAT3* knock-down induces apoptosis and cell cycle block in human TEL-AML1$^+$ cell lines.

The figure show Western blot analysis of STAT3 silencing in Reh (**A**) and At-2 (**B**) 5 days after transduction with control scramble or two different STAT3 shRNAs. Numbers represent densitometric quantitation of STAT3 bands normalized to GAPDH. The bar charts represent percentage of apoptotic Annexin V^+PI^- and percentage of cells in the S-phase of the cell-cycle six days after transduction with control scramble or STAT3 shRNAs in Reh (**C**) and At-2 (**D**). Cells were pulsed with 10μM 5-ethynyl-2´-deoxyuridine (Edu) for 1 hour. All data are representative of three independent experiments and show means ± s.d. of triplicate measurements. **P* < 0.05, ***P* < 0.01, ****P* < 0.005 compared to control (Student's unpaired *t* test).

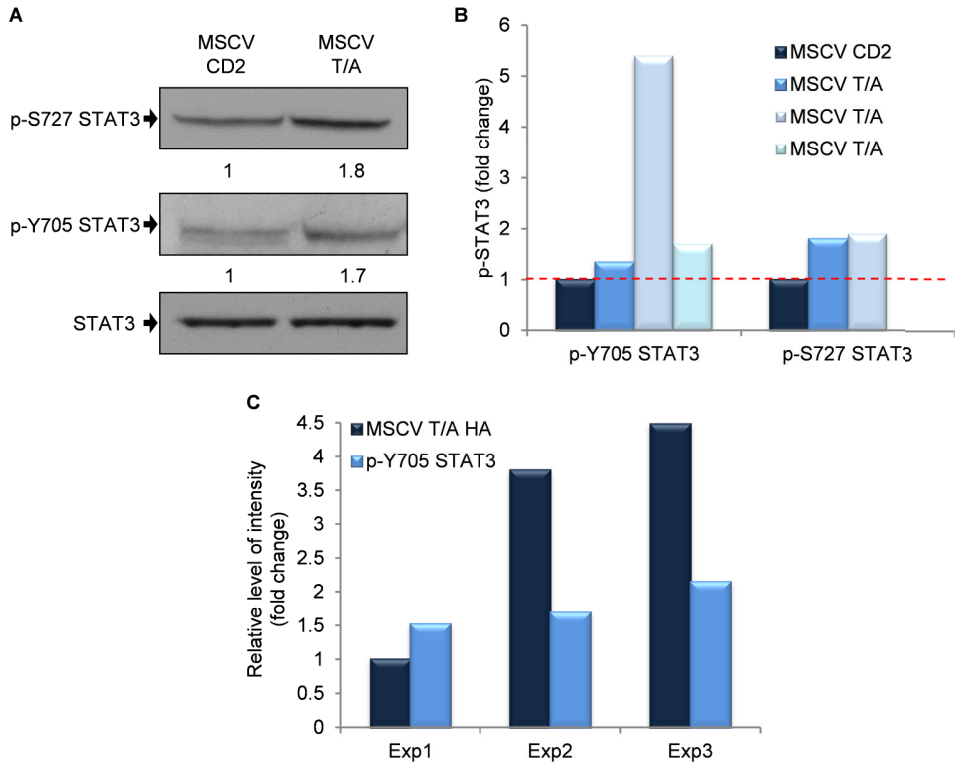


Figure 4-10: Over-expression of TEL-AML1 in HPCs induces STAT3 phosporylation.

The figure shows western blot analysis of STAT3 Y705 and STAT3 S727 in mouse c-kit^{+} Ter119^{-} foetal liver haematopoietic progenitor cells (HPCs), 72 hours after transduction with TEL-AML1 expressing (MSCV T/A) or empty (MSCV CD2) retroviral vectors (**A**). Numbers represent densitometric quantitation of p-STAT3 bands normalized to total STAT3. (**B**) Bars represent the densitometric quantitation of three independent experiments (MSCV T/A) for p-Y705 STAT3 and two for p-S727 STAT3, in each case normalised to vector control (MSCV CD2). p-STAT3 bands are normalized to total STAT3. (**C**) Due to variability of the STAT3 Y705 induction three more experiments were performed to compare the phosphorylation level to TEL-AML1

expression. Bars represent the densitometric quantitation of the relative expression levels of HA-tagged TEL-AML1 (normalized to levels expressed in experiment 1) and fold induction p-Y705 STAT3 in TEL-AML1 versus empty vector transduced cells (normalized to total STAT3).

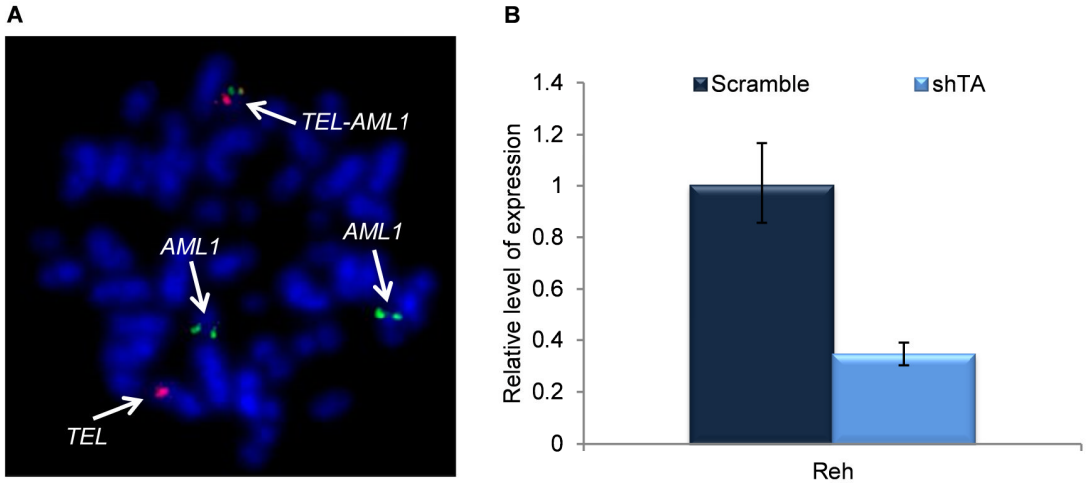


Figure 4-11: shRNA-mediated TEL-AML1 knock-down.

The image shows the loss of the second allele of *TEL* in Reh (**A**). Red probe: *TEL*, green probe: *AML1* (Analysis performed by Irina Stasevich). The bar chart shows the level of knock-down of *TEL-AML1* in Reh analysed by qRT-PCR (**B**) using a probe detecting the region of *TEL* still present in the fusion. Data are representative of three independent experiments and show means ± s.d. of quadruplicate measurements.

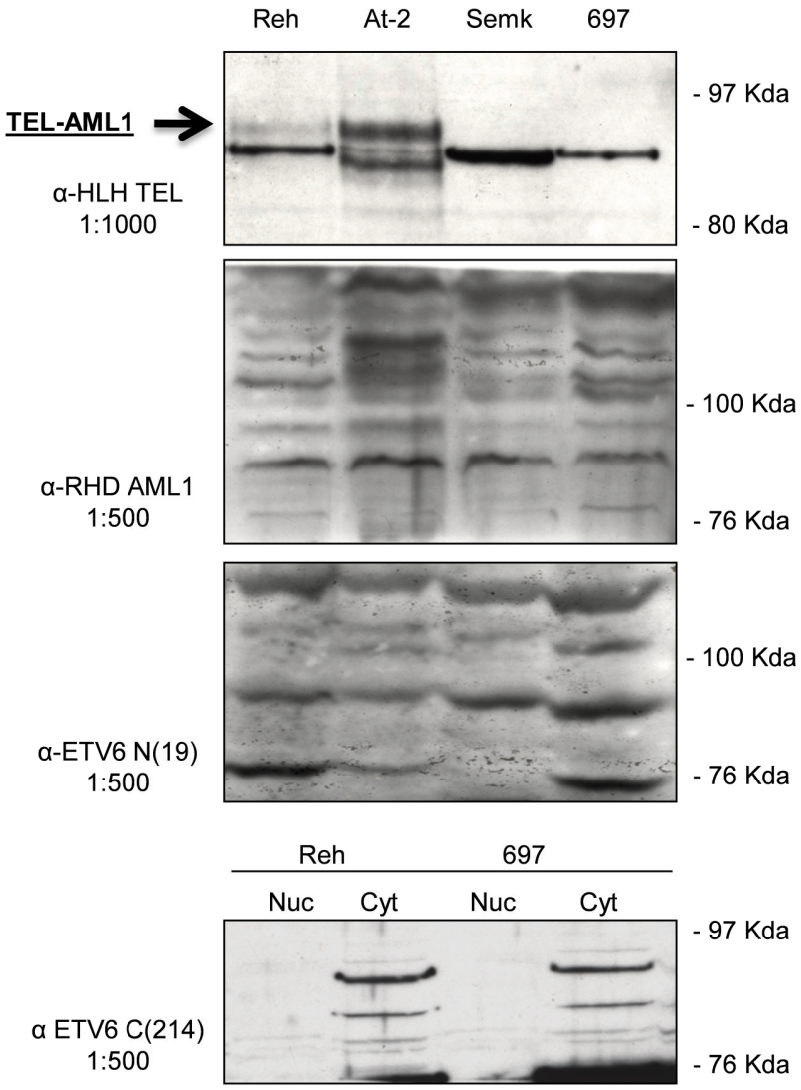


Figure 4-12: TEL-AML1 protein identification by western blot.

The image shows the western blot analysis of nuclear and cytoplasmic lysates from human leukemic cell lines positive (Reh and At-2) and negative (Semk and 697) for the presence of TEL-AML1. Different antibodies, indicated beside each blot, were tested in order to identify the endogenous fusion protein (predicted size ~90kda).

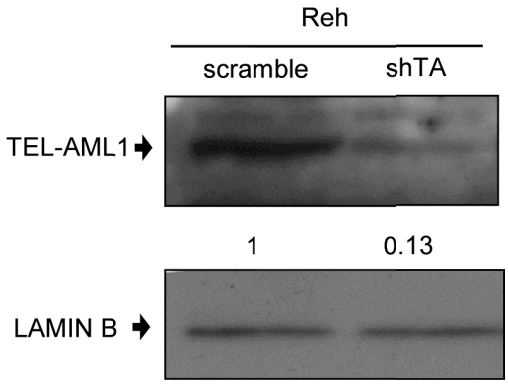


Figure 4-13: Protein analysis of *TEL-AML1* knock-down.

The figure displays the level of knock-down of TEL-AML1 protein in Reh using nuclear extract six days after transduction with an shRNA against *TEL*. Numbers represent densitometric quantitation of TEL-AML1 bands normalized to nuclear control LAMIN B. Data are representative of three independent experiments.

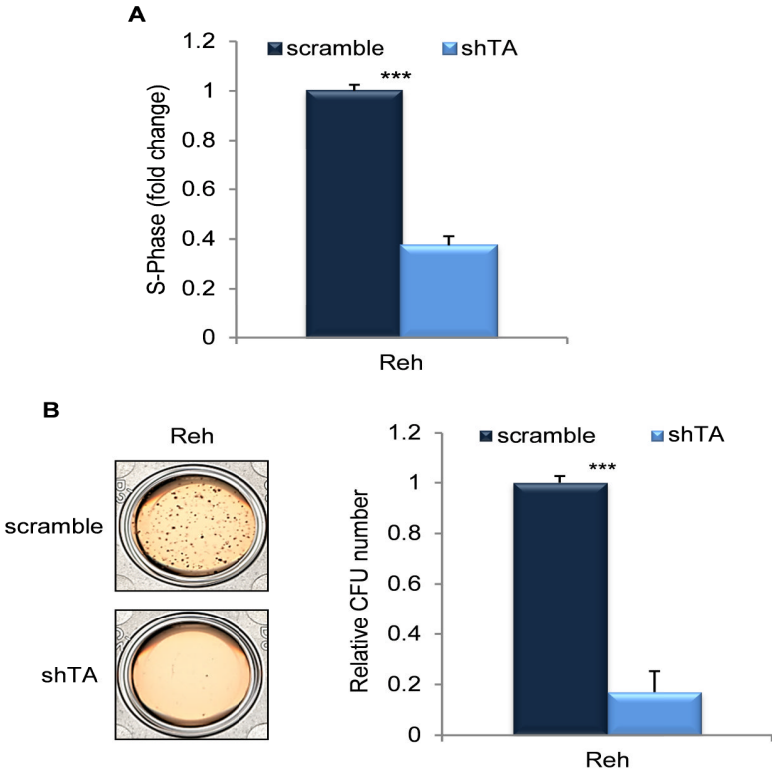


Figure 4-14: *TEL-AML1* silencing induces cell cycle block and reduces self-renewal ability.

The bar graphs show the percentage of cells in the S-phase of the cell-cycle six days after transduction with control scramble or shTA shRNA in Reh cells (**A**). Cells were pulsed with 10μM 5-ethynyl-2´-deoxyuridine (Edu) for 1 hour. Colony formation in methylcellulose by Reh cells transduced with control scramble or shTA shRNA was also evaluated (**B**). Bars show the number of colonies formed relative to control cultures. The cells were plated at 0.18×10^4 cells per well in a 24 multiwell plate and stained with INT after 14 days. All data are representative of three independent experiments and show means ± s.d. of triplicate measurements. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ compared to control (Student's unpaired *t* test).

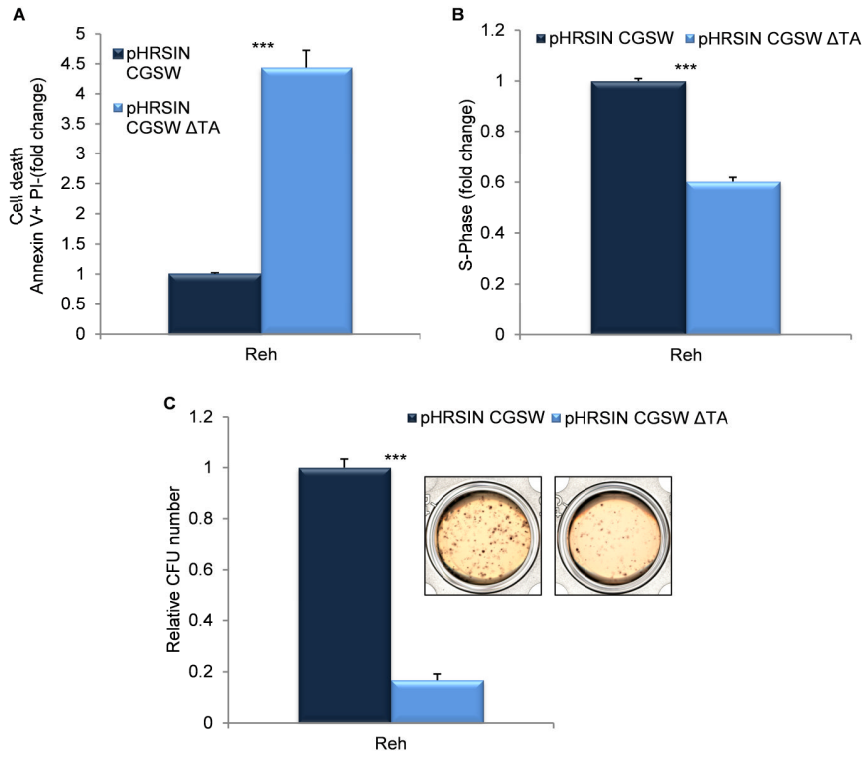


Figure 4-15: Overexpression of a TEL-AML1 mutant causes cell death, cell cycle block and impairs self-renewal ability in Reh.

The bar charts represent the percentage of apoptotic Annexin V^+PI^- cells (**A**) and the percentage of cells in the S-phase of the cell-cycle (**B**), six days after transduction of Reh cells with control vector (pHRSIN CGSW) or TEL-AML1 mutant (pHRSIN CGSW ΔTA). Cells were pulsed with 10μM Edu for 1 hour. CFU formation by Reh cells transduced with control or ΔTA was also evaluated (**C**). Bars show the number of colonies formed relative to control cultures. An example of colony formation is also shown. The cells were plated at $0.18x10^4$ cells per well in a 24 multiwell plate and stained with INT after 14 days. All data are representative of three independent experiments and show means ± s.d. of triplicate measurements. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$ compared to control (Student's unpaired *t* test).

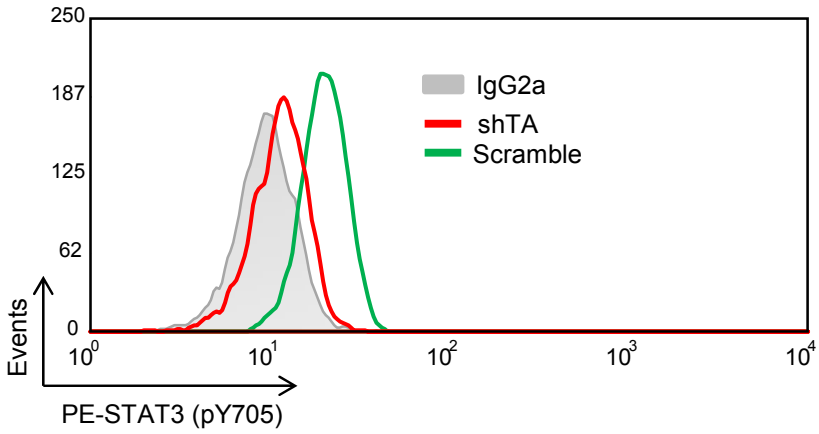


Figure 4-16: STAT3 Y705 level is reduced as consequence of *TEL-AML1* silencing.

The image shows FACS analysis of p-Y705 STAT3-PE in Reh 6 days after transduction with control scramble (MFI=21.04) or shTA (MFI=12.01) shRNA. Isotype control staining (MFI=10.23) is also shown. All data are representative of three independent experiments.

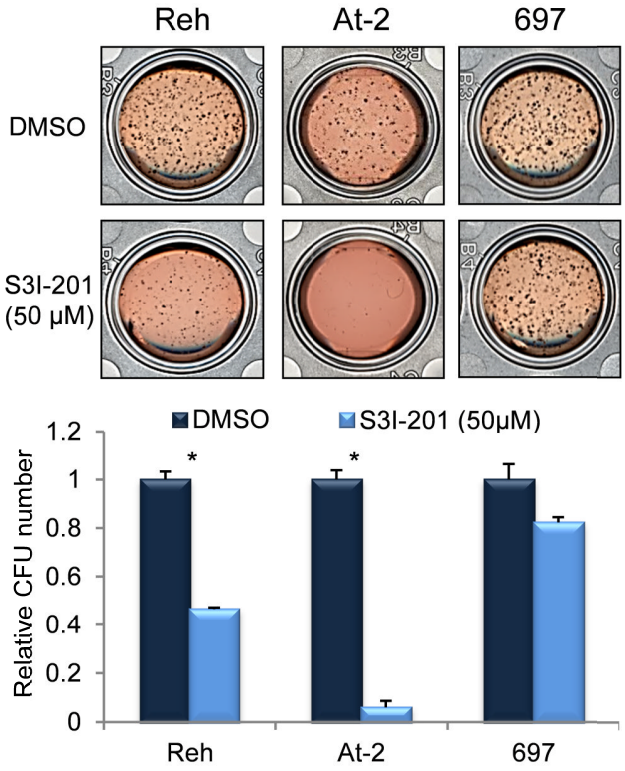


Figure 4-17: Pharmacological inhibition of STAT3 impairs the self-renewal ability of TEL-AML1 luekaemic cell lines.

The figure shows the colony forming ability of TEL-AML1 and 697 leukaemic cells upon exposure to S3I-201 in methylcellulose based media. The cells were plated at $0.18x10^4$ cells per well in a 24 multiwell plate and stained with INT after 14 days. Quantification of the number of colonies obtained after DMSO or S3I-201 treatment is also shown. All data are representative of three independent experiments and show means ± s.d. of duplicate measurements. **P* < 0.05, ***P* < 0.01, ****P* < 0.005 compared to control (Student's unpaired *t* test).

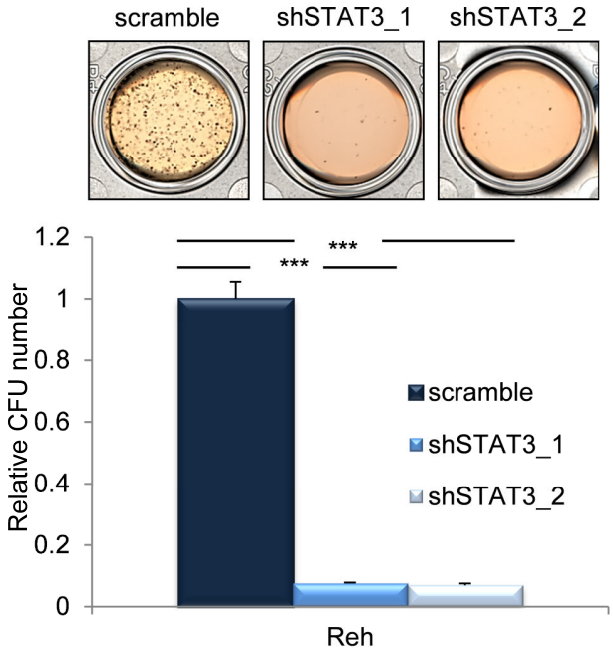


Figure 4-18: shRNA mediated *STAT3* knock-down reduces self-renewal ability of Reh cells.

The figure shows the colony forming ability of Reh cells following *STAT3* silencing in methylcellulose based media. The cells were plated at $0.18x10^4$ cells per well in a 24 multiwell plate 5 days after lentiviral transduction and stained with INT after 14 days. Quantification of the number of colonies obtained is also shown. All data are representative of three independent experiments and show means ± s.d. of triplicate measurements. *P < 0.05, **P < 0.01, ***P < 0.005 compared to control (Student's unpaired t test).

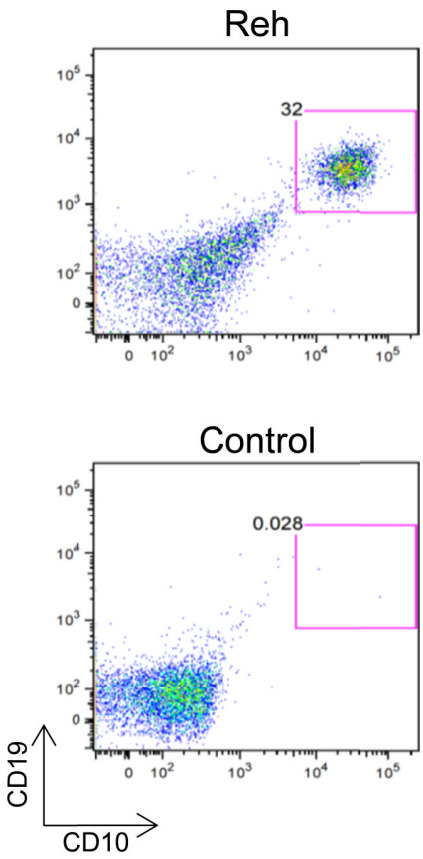


Figure 4-19: Reh engraftment in NSG mice.

Flow cytometry plots demonstrating the presence of human leukaemic cells in the bone marrow of mice transplanted with Reh. Bone marrow cells from NSG control mice and mice transplanted with 10^5 human leukaemic cells were collected and analyzed by flow cytometry. Engraftment is defined as the presence of human $CD10^+CD19^+$ cells in the bone marrow (32% in Reh transplanted mice). The mice were sacrificed after developing signs of disease (loss of movement, hind limb paralysis).

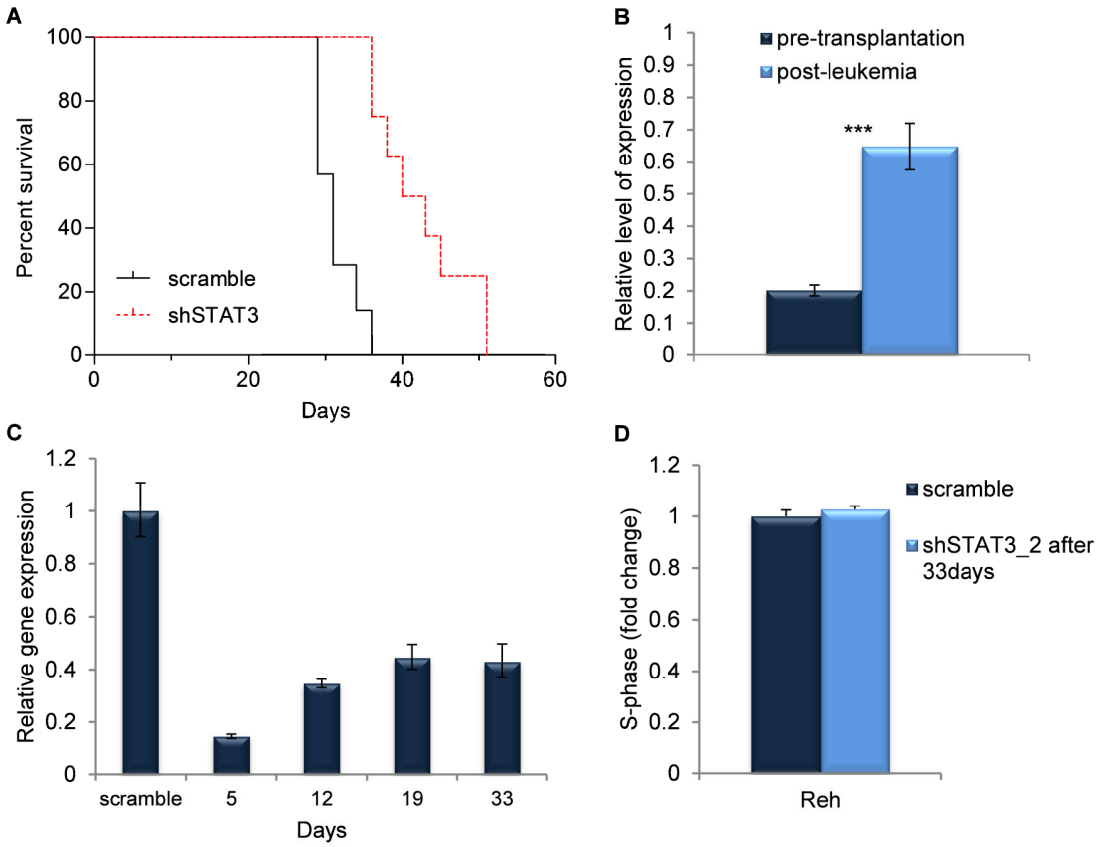


Figure 4-20: Leukaemia progression *in vivo* after *STAT3* silencing.

The figure shows the Kaplan-Meier survival curve for NSG mice transplanted with control scramble (n=7) or shSTAT3 (n=8) transduced Reh cells. *p*=0.002 versus scramble control (Mantel-Cox logrank test) (**A**). Although all the mice succumbed to leukaemia, qRT-PCR analysis of *STAT3* mRNA expression in Reh cells prior to transplantation (pre-transplantation: 6 days after transduction with shSTAT3) and in shSTAT3 transduced cells isolated from bone marrow of leukaemic mice (post leukemia) showed loss of knock-down after *in vivo* progression (**B**). Columns represent the mean ± s.d. of measurements for two independent transduction experiments (before) and three independent leukaemic mice (after). *STAT3* expression is normalized to *STAT3* expression

in control scramble shRNA transduced Reh cells. ***$P < 0.005$ compared to control (Student's unpaired *t* test). *STAT3* mRNA was also followed *in vitro* for 33 days (**C**), demonstrating a loss of silencing that resulted in similar percentages of cells being present in the S-phase of the cell-cycle (**D**). (**C**-**D**) Columns represent the mean ± s.d. of triplicate measurements.

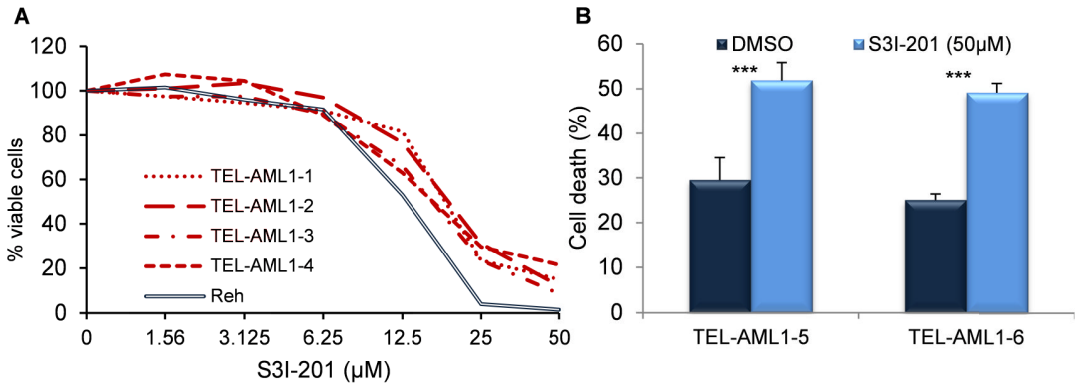


Figure 4-21: Primary TEL-AML1 samples are sensitive to S3I-201 .

The chart shows the proliferation of primary human TEL-AML1 leukaemic cells as measured by MTS assay after 96 hours culture with the indicated concentrations of the S3I-301 inhibitor (**A**). The Reh cell line was used as a reference. Results are normalized to the proliferation of DMSO treated cells. The bar chart represents the percentage of total cell death of two primary samples measured by Annexin V PI staining after 24 hours of culture with 50μM S3I-301 (**B**). All data show means ± s.d. of triplicate measurements. **P* < 0.05, ***P* < 0.01, ****P* < 0.005 compared to control (Student's unpaired *t* test).

CHAPTER V: RAC1 regulates STAT3 activity in TEL-AML1 leukaemia

5.1 Introduction

5.1.1 How is STAT3 activated in t(12;21) ALL?

Cell survival, cell proliferation and cell differentiation are often regulated by extracellular factors that can be grouped into three different categories: cytokines, hormones and growth factors. Cell surface receptors can be activated by all three of these different elements and can mediate signals from the extracellular environment to the nucleus, coordinating responses through different molecular signalling networks. Several of these signal transduction cascades culminate in the activation of STAT proteins. Classically, ligand induced receptor activation causes STAT binding to phosphotyrosine residues of the stimulated membrane receptor, through their SH2 domains, and subsequent tyrosine phosphorylation of the STAT proteins by the receptor itself or by associated kinases.

There are many ligands capable of activating STAT proteins. Some, such as the epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and colony-stimulating factor–1 (CSF1) are ligands for receptors with intrinsic tyrosine kinase activity. In contrast, others bind receptors that lack intrinsic tyrosine kinase activity but are capable of binding to Janus kinases (JAKs) that are responsible for STAT protein activation. This is the case for the large haematopoetin subfamily of cytokines that includes the interferons (IFN-α,

-β and –γ) and interleukins 2-7, 10-13, 15, 19-24, 27 and 35 (Darnell, 1997; Schindler and Plumlee, 2008). Binding of these cytokines to their receptors leads to the activation of JAKs, which then phosphorylate themselves and the bound receptors at specific tyrosine residues which can be recognized by the SH2-domains of STAT proteins, or other SH2-containing adaptor proteins (Imada and Leonard, 2000).

TEL-AML1^{+} leukaemia cells have never been associated with a dependence upon particular growth factors or cytokines for their proliferation, and there have been no reports of growth factor over-expression thus far. There are, however, several alternative mechanisms that could account for STAT3 activation in t(12;21) ALL, such as intracellular overexpression of mediators of STAT3 signalling.

5.1.2 JAK2 and STAT3 activation

The JAK family has four different members: JAK1, JAK2, JAK3 and Tyrosine kinase 2 (TYK2) (O'Shea et al., 2004). All the members are fundamental to modulate and mediate extracellular signals into the cell. However, knock-out studies suggest they have non-overlapping functions. Thus, although JAK1-deficient and JAK2-deficient mice die prenatally, the latter due to profound anaemia (Sansone and Bromberg, 2012), JAK3-deficient and TYK-2-deficient mice are viable, exhibiting haematopoietic defects in lymphoid development and impaired cytokine responsiveness, respectively (Karaghiosoff

et al., 2000; Nosaka et al., 1995; Thomis et al., 1995). All the proteins of this family have seven domains (JH1-JH7), including a FERM (a band four-point-one, ezrin, radixin, moesin) domain for cytokine receptor interaction, an SH2 domain for protein interaction, a C-terminal kinase domain (JH1) and a pseudokinase domain (JH2). In order to transmit signals from the outside of the cell to the nucleus, JAK proteins need to be activated by auto-phosphorylation at specific tyrosine residues. This phosphorylation is triggered by ligand-induced conformational changes in receptors, resulting in juxtaposition of receptor-bound JAK molecules. These then activate each other by trans-phosphorylation, and subsequently phosphorylate the ligand-bound receptors and downstream targets, such as the STAT proteins. One of the main JAK family members responsible for STAT3 activation in different models and cancers is JAK2 (Behera et al., 2010; Marotta et al., 2011).

Constitutive activation of the JAK/STAT pathway has been described in many diseases and particularly in haematological cancers. JAK2 mutations that cause constitutive STAT protein activation, and thereby confer growth factor-independent proliferation of malignant clones, are frequent in myeloproliferative neoplasms (Kralovics et al., 2005). They have been identified also in B-cell ALL, particularly when this is associated with Down's syndrome (Mullighan et al., 2009). In this context, mutations of JAK2 are often associated with overexpression of the CRLF2 receptor that leads to an even more powerful activation of the JAK-STAT pathway (Harvey et al., 2010). Additionally, in some patients with T-cell ALL, pre-B-cell ALL and atypical CML, JAK2 has been

shown to be involved in the formation of a fusion protein with TEL, which is responsible for the constitutive activation of STAT1, STAT3 and STAT5 (Lacronique et al., 1997).

5.1.3 Intracellular activation of STAT3: the role of RAC1

As previously mentioned, besides JAK-mediated activation, STAT3 can also be phosphorylated by different cytoplasmic tyrosine kinases and by growth-factor receptors that have intrinsic tyrosine-kinase activity. The first category includes SRC-related kinases, Abelson leukaemia protein (ABL) and c-Fes protein, whereas epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR) and hepatocyte growth factor (HGF) all belong to the second. Recent evidence indicates that members of the small Rho GTPase family are also able to activate STAT3. The Rho (Ras homologous) proteins are a subgroup of the Ras small GTPase superfamily that differ from the other members of the family by the presence of a Rho-specific insert in the GTPase domain. More than twenty Rho GTPases have been described but only 3 members, RhoA, Rac1 and Cdc42 are ubiquitously expressed. The main function of these proteins is the regulation of cytoskeleton remodelling and they are all involved in actin polymerization, cell polarization, motility and adhesion. In addition, they also function in cell growth, apoptosis, cell cycle progression and genomic stability (Bokoch, 2000). For these reasons, deregulation of their expression can play a role in oncogenesis and

tumour progression. Thus, increases in the levels of Rho family proteins have been observed in a number of cancers (Karlsson et al., 2009).

There are three different isoforms of the RAC subfamily that are differentially expressed: RAC1, RAC2 and RAC3. RAC1 and RAC3 are more widely expressed than RAC2, which is restricted to cells of haematopoietic origin. The most studied factor of the family is RAC1 and it has emerged as a fundamental upstream activator of multiple signaling pathways regulating gene expression, including STAT3. However, the mechanisms by which RAC1 is able to activate STAT3 remain unclear. Some studies suggest that RAC1 activates STAT3 directly, reporting binding between the two proteins (Simon et al., 2000), but evidence also exists for indirect induction via IL-6/JAK signaling (Debidda et al., 2005; Faruqi et al., 2001). RAC1 has also been shown to form a complex with STAT3 and MgcRacGap, a RAC1 GTPase activating protein. This association was shown to be required for STAT3 phosphorylation and nuclear translocation (Kawashima et al., 2006; Tonozuka et al., 2004) (Figure 5-1).

5.1.4 Regulation of RAC1 activity

The activity of RAC1 is mainly regulated by two groups of proteins, called Guanine nucleotide exchange factors (GEFs) and GTPase-Activating Proteins (GAPs). The proteins belonging to these two families are able to regulate the GTP/GDP cycle and consequently the levels of active and inactive

RAC1 within the cell. Furthermore, the activity of RAC1 is also negatively regulated by another group of proteins, specific for the RHO GTPase family, called Guanosine nucleotide dissociation inhibitors (GDIs) (Figure 5-2).

RAC1 activation is mediated by GEF proteins, which are responsible of converting the inactive GDP-bound form of RAC1 to the active GTP-bound form. The latter is able to interact with specific effectors, such as p21 activating kinases (PAK) (Frost et al., 1996; Manser et al., 1994) and MLK2/3 (Teramoto et al., 1996), and in this way to coordinate the activation of a multitude of signaling pathways that regulate different cellular processes. Most of the GEFs able to activate RAC1 are characterized by a Dbl-homology (DH) domain, that is responsible of the catalytic activity of the proteins, and a pleckstrin homology (PH) domain, which affects the activity of the DH domain and is responsible for the localization of the GEF proteins to the plasma membrane, through lipid binding (Karnoub et al., 2001). The activity of some of the GEF proteins appears to be highly specific towards a single GTPase, whereas others are reported to be able to activate several GTPases. Moreover, differences have also been reported between *in vitro* and *in vivo* GEF specificities (Schmidt and Hall, 2002). GEF activation is tightly regulated through three different mechanisms: relief of intramolecular inhibitory sequences by allosteric modification induced mainly by phosphorylation, stimulation and inactivation by protein-protein interactions, and alteration of intracellular localization (reviewed in (Schmidt and Hall, 2002)).

In contrast, GAP proteins function by terminating the signalling event through increasing the rate of GTP hydrolysis and consequent conversion of GTP-bound to GDP-bound GTPase. However much less is known about this process. Additionally, the proteins of the Rho family are negatively regulated by the action of Rho guanine nucleotide dissociation inhibitors (Rho-GDIs) (Mackay and Hall, 1998). These proteins regulate GTPase signalling by three different mechanisms: they can prevent nucleotide dissociation and GDP/GTP exchange to antagonize GEF activity, interfere with GTP hydrolysis and stimulate the release of Rho GTPases from the membrane, where they are active, into the cytosol (Karnoub et al., 2004).

5.2 Results

5.2.1 Analysis of JAK2 in TEL-AML1 leukaemia.

Based on the association of JAK2 with STAT3 activation in the literature, we decided to examine whether JAK2 was responsible for the constitutive activation of STAT3 in TEL-AML1 leukaemia. We tested the JAK2 inhibitor AG490 on our panel of pre-B cell lines, in a similar assay to that used to demonstrate the inhibitory activity of S3I-201. However, in contrast to S3I-201, AG490 did not have any inhibitory effect on the proliferation and survival of TEL-AML1$^+$ cells. In contrast, the BCR-ABL$^+$ TOM-1 cells exhibited sensitivity to JAK2 inhibition, as previously reported (Deininger et al., 1997). These data

suggest that an alternative pathway may be responsible for STAT3 activation in TEL-AML1 leukaemia (Figure 5-3).

5.2.2 Role of RAC1 in TEL-AML1 leukaemia

Based on the evidence that RAC1 is a mediator for the activation of STAT3 and that Reh cell lines were shown to be highly sensitive to RAC1 pharmacological inhibition (Troeger, 2010), we decided to examine whether the activation of STAT3 observed in TEL-AML1 leukemia could be mediated by RAC1.

For this purpose we measured the level of Tyr705 STAT3 in Reh and At-2 cells six hours after treatment with the RAC1 inhibitor NSC 23766. This inhibitor acts by blocking the binding of RAC1 protein to the specific activating GEFs TrioN and Tiam 1 (IC_{50} = 50 μM) showing no effect on the closely related GTPases, Cdc42, and RhoA (Gao et al., 2004). Both Western blot (Figure 5-4A) and flow cytometric analysis (Figure 5-4B) demonstrated a dose-dependent inhibition of Tyr705 STAT3 phosphorylation in response to NSC 23766, suggesting a role for RAC1 in regulating the activation of STAT3 in these cells. In addition, in order to assess the importance of RAC1 for the survival of TEL-AML1$^+$ cells, we measured the level of apoptosis and cell cycle progression after NCS 23766 treatment. Exposure of both TEL-AML1$^+$ human leukaemic cells to 50μM of the compound for 24 hours resulted in induction of a similar percentage of cell death (Figure 5-5A) and cell cycle block (Figure 5-5B)

to that observed with the STAT3 inhibitor S3I-201. We showed previously, that TEL-AML1 is capable of activating STAT3 in mouse HPC. In order to determine whether RAC1 induction was similarly dependent on TEL-AML1, we examined RAC1 activity in mouse HPC following transduction with the fusion gene. Indeed, TEL-AML1 overexpression resulted in a marked increase in the levels of GTP-RAC1 present in the cells (Figure 5-6A). Furthermore, this dependence was conserved in human leukaemia cells. Silencing of fusion gene expression in Reh cells, using the shRNA against TEL, also resulted in reduced GTP-RAC1 levels (Figure 5-6B).

Taken together, these data suggest a role for TEL-AML1 in regulating the activity of RAC1, which in turn induces STAT3 activation.

5.2.3 RAC1 activation is mediated by ARHGEF4 in TEL-AML1 leukaemia

Activation of RAC1 in TEL-AML1 leukemia cell lines has been reported also by other groups (Holland et al., 2011). However, how this activation is achieved has not yet been clarified. One possible mechanism could be via up-regulation of RAC1-specific guanine nucleotide exchange factor (GEF) expression. A recent study from our group showed that the RAC1/CDC42 GEF *ARHGEF4* (*ASEF*) is specifically associated with TEL-AML1 (Lyons et al., 2010) in gene expression datasets of childhood acute leukaemias (Haferlach et al., 2010; Ross et al., 2003; van Delft et al., 2005) (Figure 5-7). This GEF was first identified by Thiesen et al. from a human neuronal teratocarcinoma

library screen (Thiesen et al., 2000). Subsequently, ARHGEF4 was found to be regulated by the Adenomatous polyposis coli (APC) protein and hence became more commonly referred to as *ASEF* (APC-stimulated guanine exchange factor). There are two transcripts of the *ARHGEF4* gene. The two isoforms have identical sequences, apart from an additional exon in the long isoform, encoding additional C-terminal amino acids that do not contribute to the known functional domains (Thiesen et al., 2000).

The association of elevated *ARHGEF4* expression with t(12;21) ALL suggested that this gene may be a transcriptional target of TEL-AML1. In order to examine this possibility, we analysed *ARHGEF4* expression following silencing of fusion gene expression. Indeed, knock-down of *TEL-AML1* in human cell lines resulted in reduced levels of *ARHGEF4* expression, suggesting that TEL-AML1 does regulate the expression of this GEF in human leukaemia cells (Figure 5-8). In order to examine whether ARHGEF4 regulates the activity of RAC1 in these cells, we measured the levels of GTP-RAC1 in Reh cells three days after shRNA-mediated silencing of *ARHGEF4* gene expression. Despite only achieving moderate silencing of *ARHGEF4*, this was sufficient to cause a significant reduction in the activity of RAC1 (Figure 5-9A and B). This reduction, moreover, resulted in decreased levels of Tyr705 STAT3, confirming our previous data that indicated a dependence of STAT3 phosphorylation on RAC1 activity in TEL-AML1^{+} leukaemia (Figure 5-9C). In addition, *ARHGEF4* silencing in Reh cells resulted in almost complete cell death after 5 days (Figure 5-10A) and abrogation of their ability to form

colonies in methylcellulose (Figure 5-10B). Finally, we decided to transplant NSG mice with Reh cells silenced for *ARHGEF4*. We injected the cells three days after transduction with shRNA, thus keeping the resultant apoptosis induction to a minimum, allowing us to compare engraftment with equivalent numbers of viable control and *ARHGEF4*-silenced cells. None of the mice transplanted with Reh cells deficient for *ARHGEF4* expression developed leukaemia. In contrast, mice transplanted with an equivalent number of viable scramble-shRNA transduced Reh cells all succumbed to leukaemia (Figure 5-11). These data indicate that ARHGEF4 expression is critical for the *in vivo* engraftment of TEL-AML1^{+} cells, and suggest that this GEF plays a fundamental role in the progression of TEL-AML1^{+} ALL.

5.3 Discussion

As already mentioned, the aberrant activation of STAT3 associated with numerous different cancers can be achieved in many different ways. Here we show that in TEL-AML1$^+$ leukaemia this activation is induced mainly by RAC1. Although RAC1-mediated STAT3 activation has previously been reported in a number of different studies, the mechanism remains unclear. Some studies have reported that RAC1 activates STAT3 directly, but evidence also exists for indirect induction via interleukin 6 signalling. The insensitivity of TEL-AML1$^+$ cell lines to JAK2 inhibition in our experiments suggests that the activation of STAT3 by RAC1 in these cells is likely to occur through a direct intracellular mechanism. However, other intracellular kinases, such as other JAKs or SRC and SYK family members, could also be responsible for STAT3 activation, in cooperation with or independent of RAC1. Further experiments are therefore required in order to clarify the mechanisms underlying STAT3 activation in these cells.

RAC1 plays an important role in haematopoiesis and normally is coexpressed with RAC2 in cells of haematopoietic origin. This coexpression is likely to result in a degree of functional redundancy, with potentially overlapping functions (Weston and Stankovic, 2004). Analysis of TEL-AML1$^+$ cell lines, however, did not reveal any significant RAC2 activity, although primary samples were not analysed in this study, suggesting a unique role for RAC1 in this subtype of leukaemia (Holland et al., 2011). Activity of RAC1 is mainly modulated by GEFs and GAPs, which are able to regulate the transition

between the inactive GDP-bound form to the active GTP-bound state and vice versa. Alterations in the function of these factors, has been demonstrated to play a role in cancer development (Vigil et al., 2010). In contrast to the RAS GEF family, no point mutations have been reported in the RHO GEF family. However, their overexpression has been implicated in the growth of several cancers. In addition, two RHO GEFs are structurally mutated in leukaemia by chromosome rearrangement, giving rise to the mixed-lineage leukemia (MLL) - ARHGEF12 (Kourlas et al., 2000) fusion protein and BCR-ABL1 fusion protein (Sahay et al., 2008), both of which retain the ability to activate RHO proteins. In contrast to the many GEFs reported to be altered in cancer, much less is known about GAPs. The best characterized example is the tumour suppressor role for ARHGAP7 in many cancers (Lahoz and Hall, 2008). Deregulated GEF, GAP or GDI gene expression would be expected to contribute to the aberrant activation of RAC1 in cancer. Indeed, here we show that expression of the RAC1-specific GEF ARHGEF4 is associated with TEL-AML1 leukaemia and its elevated expression is responsible for RAC1 activation. However, *ARHGEF4* silencing resulted in only partial reduction in the activity of STAT3, suggesting that alternative pathways may also be involved in STAT3 activation in these cells. On the other hand, *ARHGEF4* silencing resulted in complete loss of cell viability. Indeed, the effect of losing expression of this GEF was even more pronounced than that resulting from STAT3 and RAC1 inhibition, suggesting that it may regulate additional signalling cascades critical for survival of TEL-AML1 leukaemia cells. However, identification of these cascades is

complicated by the limited number of studies that have addressed ARHGEF4 function. ARHGEF4 has been described as an important mediator of APC function, playing a role in the migration of colorectal tumour cells, expressing truncated APC (Kawasaki et al., 2003). It has also been shown to act as a downstream effector of hepatocyte growth factor (HGF) and phosphatidylinositol 3-kinase (PI3K) signalling (Kawasaki et al., 2003; Kawasaki et al., 2009). This may suggest that in TEL-AML1, these two factors play a role in stimulating ARHGEF4 activity, triggering additional signalling pathways independent of STAT3 activation.

Classically, as mentioned in the introduction, TEL-AML1 has been considered to be a transcriptional repressor. Some groups suggested a dominant-negative function of this fusion on RUNX1 target genes, similar to that observed with AML1-ETO (Zelent et al., 2004). Recent findings, however, have shown that TEL-AML1 may also activate gene expression, promoting the transcription of genes, bound by RUNX1 in normal haematopoiesis (Fuka et al., 2011). In a previous report, we showed that ARHGEF4 can co-operate with TEL-AML1 in promoting mouse B cell progenitor colony formation (Lyons et al., 2010). However, TEL-AML1 did not appear to regulate *Arghef4* gene expression in these mouse B cell progenitors. In contrast, here we show that expression of *ARHGEF4* is strictly dependent on the presence of TEL-AML1 in human cell lines. It remains unclear whether this is caused by direct binding of TEL-AML1 to *ARHGEF4* promoter sequences or via deregulation of downstream transcriptional regulators. The difference between the experiments

in mouse B cell progenitors and human leukaemia cells may be due to differences in regulation of gene expression by haematopoietic transcription factors in mouse and human cells. Indeed, recent genome-wide chromatin immunoprecipitation (ChIP) analyses demonstrated binding of RUNX1 to *ARHGEF4* promoter elements in human megakaryocytes (Tijssen et al., 2011) but not in mouse HPCs (Wilson et al., 2010). Since the RUNX1 DNA-binding domain is retained in the TEL-AML1 fusion protein, this suggests that *ARHGEF4* may be a direct transcriptional target of TEL-AML1 in human cells but not in mouse haematopoietic progenitor cells. This would further indicate that the TEL-AML1 fusion protein can function as a transcription factor able to promote the expression of genes necessary for leukaemic transformation and progression.

5.4 Figures

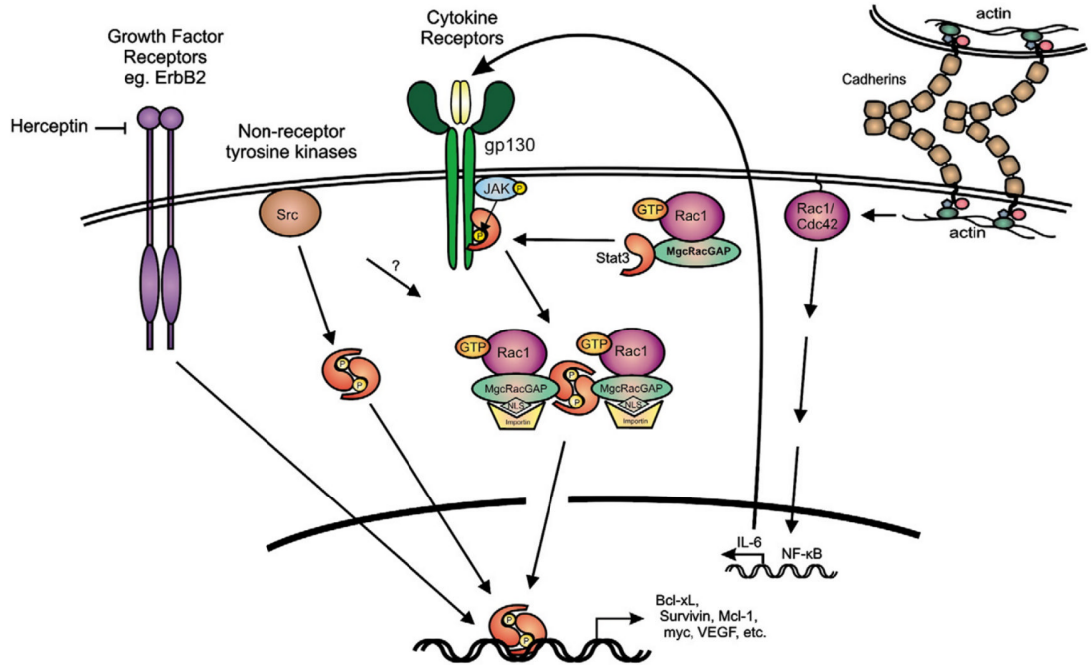


Figure 5-1: Regulation of STAT3 by RAC1 [Adapted from (Raptis et al., 2011)].

The diagram illustrates the possible mechanism for STAT3 activation mediated by RAC1. Activated RAC1 can facilitate STAT3 phosphorylation and nuclear translocation through the complex MgcRacGAP-RAC1-STAT3. In addition MgcRacGAP may also play a role in STAT3 activation by Src. RAC1 is also responsible of the activation of signalling pathways able to transcriptionally activate IL-6 production, one of the main cytokines responsible for STAT3 activation.

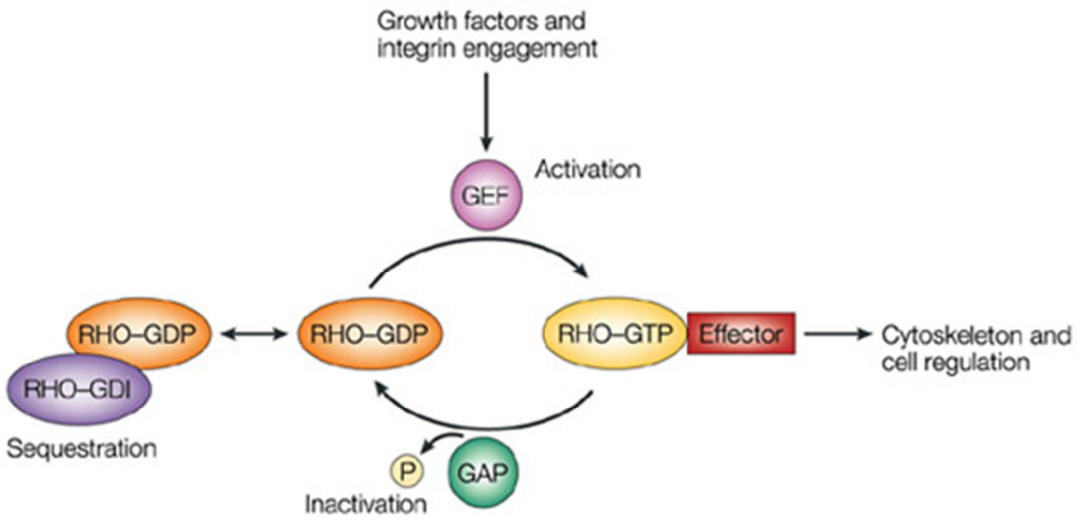


Figure 5-2: Regulation of RHO proteins activity [Adapted from (Sahai and Marshall, 2002)].

The diagram shows the regulation of the activity of RHO proteins. Upon stimulation by extracellular and intracellular factors and integrin engagement, RHO proteins are released from RHO-GDI complex and a group of proteins called GEF promote GDP-GTP exchange leading to the activation of the proteins. GAP proteins will then catalyze GTP hydrolysis allowing to GDI to reform a complex with RHO proteins locking them once again in an inactive state.

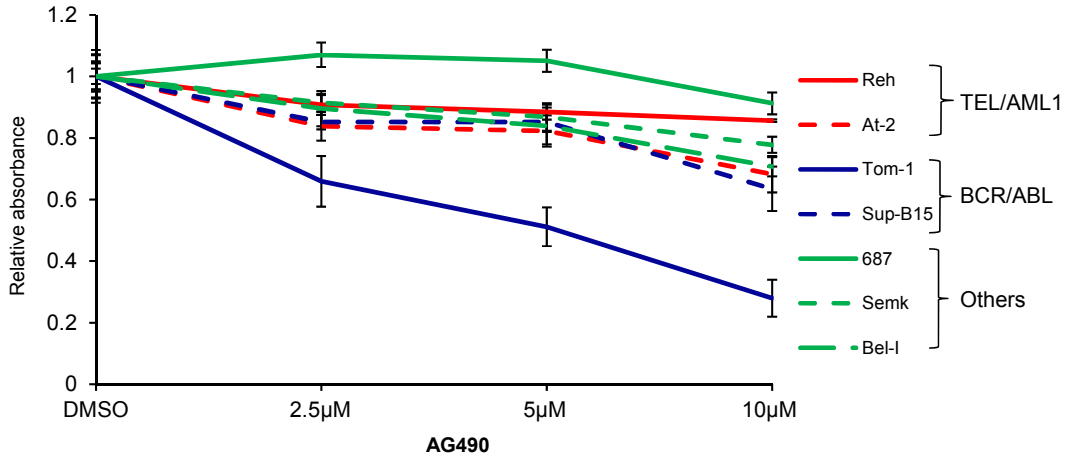


Figure 5-3: JAK2 inhibition does not affect proliferation of TEL-AML1$^+$ leukaemic cell lines.

The chart shows the proliferation of leukemic cell lines as measured by MTS assay after 96 hours culture with the indicated concentrations of a JAK2 inhibitor (AG490). Results are normalized to the proliferation of DMSO treated cells. All data are representative of three independent experiments and show means ± s.d. of triplicate measurements.

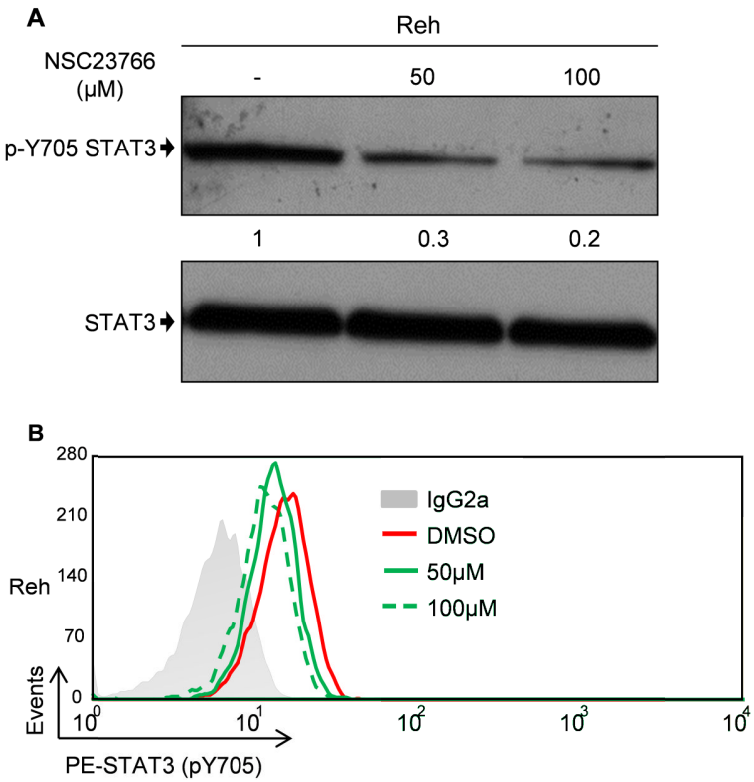


Figure 5-4: Pharmacological inhibition of RAC1 leads to reduced levels of STAT3 Y705.

The figure shows reduced levels of STAT3 Y705 after 5 hours treatment with NSC 23766 by western blot analysis (**A**) and flow cytometry analysis of p-Y705 STAT3-PE (**B**) in Reh cells. Numbers represent densitometric quantitation of p-STAT3 bands normalized to total STAT3 (**A**). The mean fluorescence intensity (MFI) of each group was as follows: 50μM (MFI=12.99) and 100μM (MFI=11.66) NSC23766 or DMSO (MFI=15.57). Isotype control staining is also shown. All data are representative of three independent experiments.

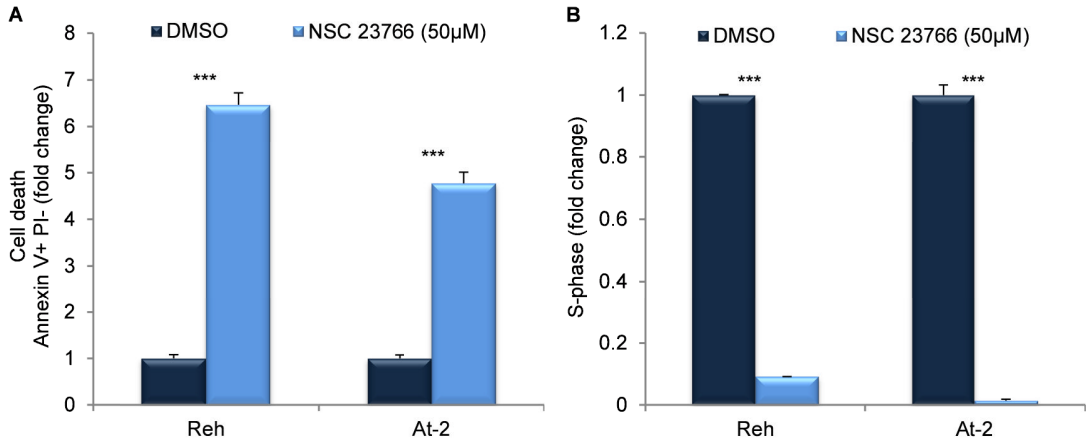


Figure 5-5: NSC 23766 treatment induces apoptosis and cell cycle block in human TEL-AML1$^+$ cell lines.

The bar charts represent percentage of apoptotic Annexin V^+PI^- (**A**) and percentage of cells in the S-phase of the cell-cycle (**B**) after 24 hours treatment with 50µM NSC 23766. Cells were pulsed with 10µM 5-ethynyl-2´-deoxyuridine (Edu) for 1 hour. All data are representative of three independent experiments and show means ± s.d. of triplicate measurements. *P < 0.05, **P < 0.01, ***P < 0.005 compared to control (Student's unpaired t test).

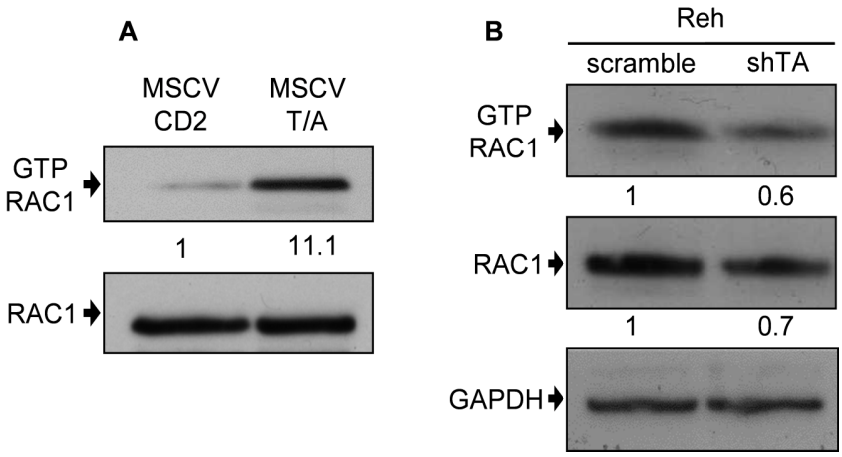
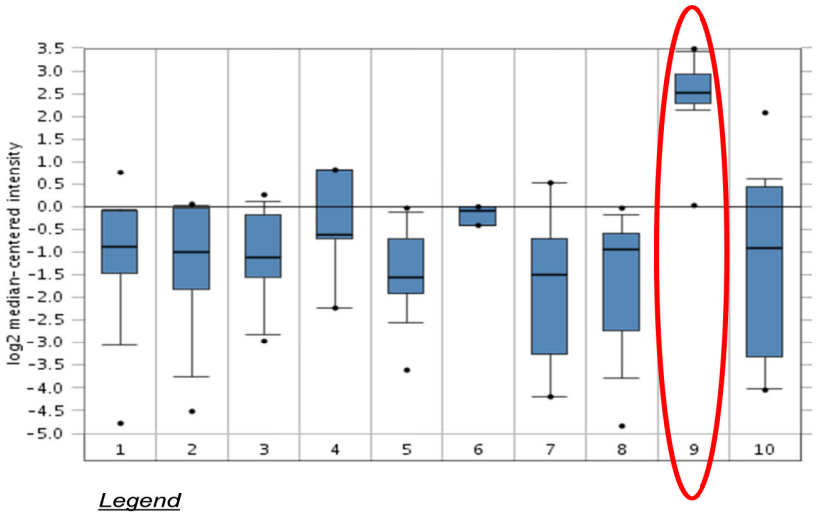


Figure 5-6: TEL-AML1 expression regulates RAC1 activity.

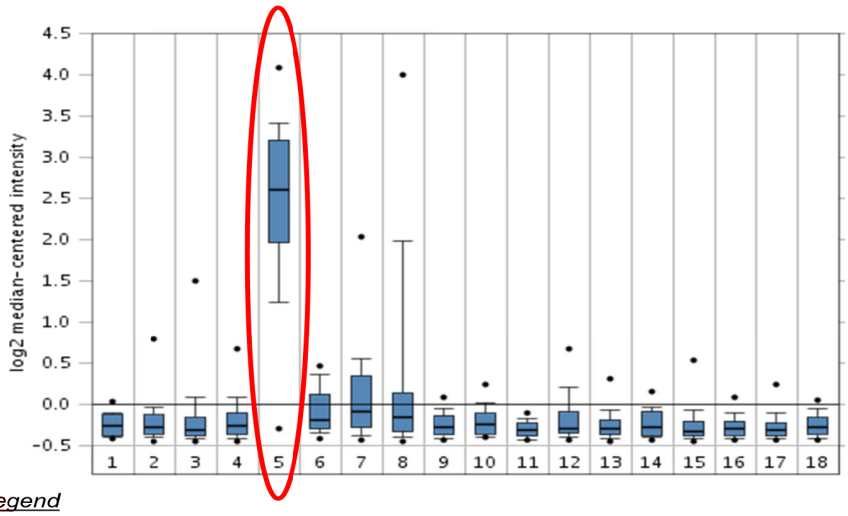
The figures show western blot analysis of RAC1-GTP pull down in mouse c-kit^{+} Ter119^{-} foetal liver haematopoietic progenitor cells (HPCs), 72 hours after transduction with TEL-AML1-expressing (MSCV T/A) or empty (MSCV CD2) retroviral vectors (**A**) and 5 days after transduction with control scramble or shTA shRNA (**B**). Numbers represent densitometric quantitation of GTP-RAC1 normalized to total RAC1 (**A**), and GTP-RAC1 and total RAC1 normalized to GAPDH (**B**). Data are representative of 3 (**A**) and 2 (**B**) independent experiments.



Legend

1. BCR-ABL (15)
2. E2A-PBX1 (18)
3. Hyperdiploid Greater Than 50 (17)
4. Hypodiploid (4)
5. MLL Rearrangement (20)
6. Normal Diploid (3)
7. Pseudodiploid (8)
8. T-ALL (14)
9. TEL-AML1 (20)
10. Novel (13)

132 samples



Legend

1. Mature B-ALL with t(8;14) (13)
2. Pro-B-ALL with t(11q23)/MLL (70)
3. c-ALL/Pre-B-ALL with t(9;22) (122)
4. T-ALL (174)
5. ALL with t(12;21) (58)
6. ALL with t(1;19) (36)
7. ALL with Hyperdiploid Karyotype (40)
8. c-ALL/Pre-B-ALL without t(9;22) (237)
9. AML with t(8;21) (40)
10. AML with t(15;17) (37)
11. AML with inv(16)/t(16;16) (28)
12. AML with t(11q23)/MLL (38)
13. AML with Normal Karyotype + Other Abnormalities (351)
14. AML Complex Aberrant Karyotype (48)
15. CLL (448)
16. CML (76)
17. MDS (206)
18. Non-Leukemia or Healthy Bone Marrow (74)

2,096 samples

Figure 5-7: Expression of *ARHGEF4* is specifically associated with t(12;21) leukaemia [Adapted from https://www.oncomine.org].

The figures show the specific association of elevated *ARHGEF4* expression with the presence of the TEL-AML1 rearrangement, after analysis of microarray expression datasets of childhood acute leukaemias. Two independent studies demonstrated that the expression of *ARHGEF4* is higher in TEL-AML1 leukaemia compared to other subtypes of ALL (Haferlach et al., 2010; Ross et al., 2003).

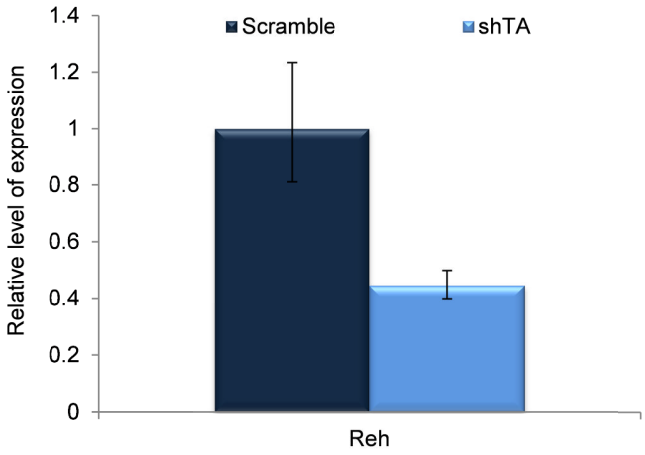


Figure 5-8: TEL-AML1 regulates the expression of *ARHGEF4* in Reh cells. The bar chart shows the down-regulation of *ARHGEF4* expression 3 days after lentiviral transduction of control scramble or shTA shRNA. All data are representative of two independent experiments and show means ± s.d. of quadruplicate measurements.

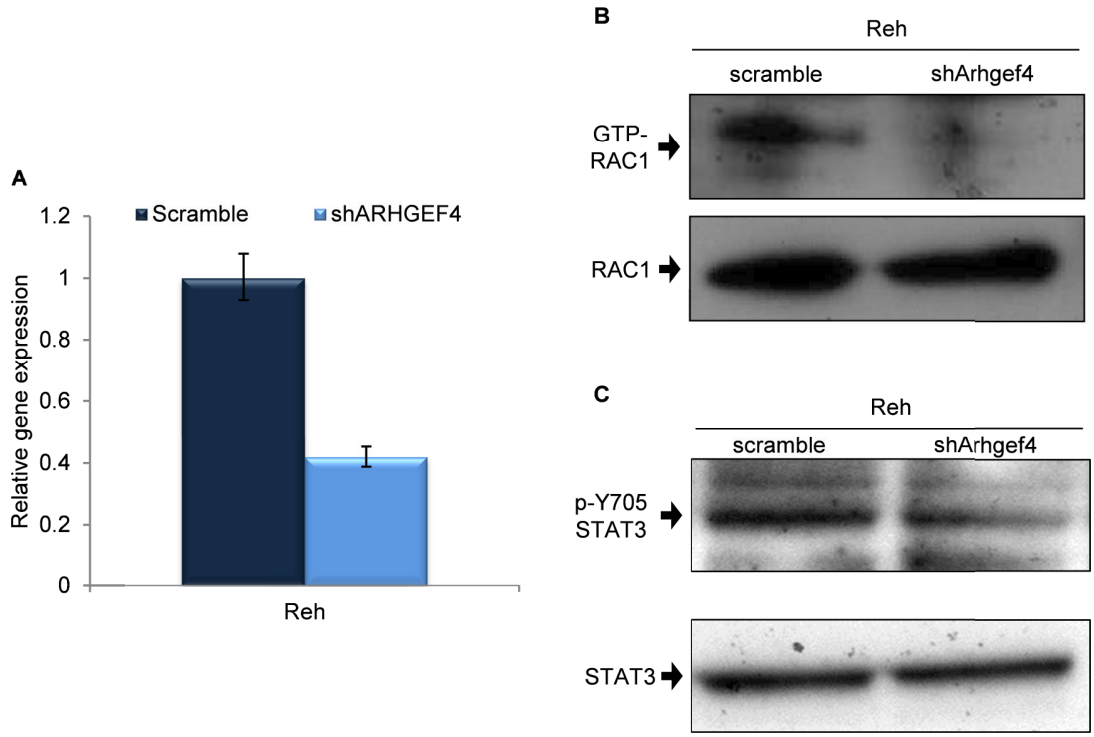


Figure 5-9: *ARHGEF4* shRNA mediated knock-down inhibits RAC1 activity and reduces STAT3 Y705 in Reh.

The figure shows *ARHGEF4* silencing in Reh (**A**) and the subsequent western blot analysis of RAC1 activity (**B**) and STAT3 phosphorylation status (**C**). The cells were analysed 3 days after lentiviral transduction to avoid loss of cell viability due to apoptosis induction. All data are representative of two independent experiments.

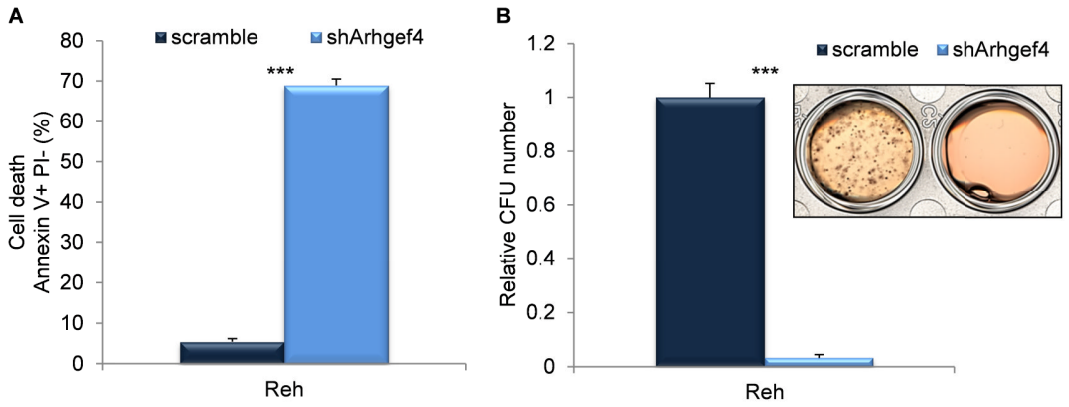


Figure 5-10: shRNA mediated silencing of *ARHGEF4* causes apoptosis and abolishes self-renewal activity in Reh cells.

The bar graphs show the percentage of Annexin V^+PI^- cells (**A**), and the colony forming ability (**B**), 5 days after transduction with control scramble or *ARHGEF4* shRNA. The cells were plated at $0.18x10^4$ cells per well in a 24 multiwell plate 3 days after lentiviral transduction and stained with INT after 14 days. All data are representative of two independent experiments and show means ± s.d. of triplicate measurements. *P < 0.05, **P < 0.01, ***P < 0.005 compared to control (Student's unpaired t test).

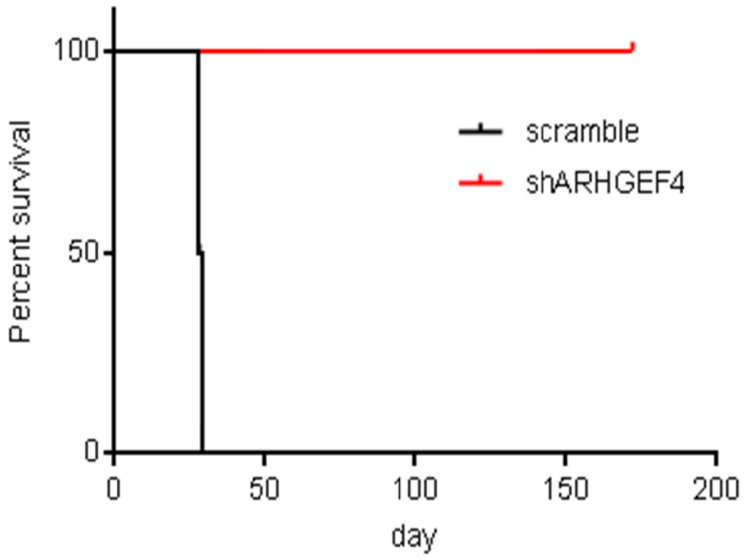


Figure 5-11: shRNA mediated *ARHGEF4* knock-down abolishes leukaemic engraftment *in vivo*.

The figure shows the Kaplan-Meier survival curve for NSG mice transplanted with control scramble (n = 4) or shARHGEF4 (n = 5) transduced Reh cells. p = 0.002 versus scramble control (Mantel-Cox log rank test).

CHAPTER VI: MYC expression is essential for TEL-AML1 leukaemia

6.1 Introduction

Classically, STAT3 has been considered to function as a transcription factor and only recently has it been shown to have a function independent of regulating gene expression. In response to activation, STAT3 is phosphorylated on Tyr-705 and forms homodimers through phosphotyrosine-SH2 domain interactions. In addition, STAT3 is able to dimerize with other STAT proteins. It has been shown, for instance, that it can bind and synergise with STAT4 in Human T-lymphotropic virus Type I (HTLV)-I-transformed T-cell lines. Under particular conditions, formation of STAT3/STAT5 and STAT3/STAT1 heterodimers has been also reported (Higashi et al., 2005; Novak et al., 1996; Stancato et al., 1996). Following dimer formation, these complexes translocate into the nucleus, bind to DNA elements within the promoter regions of specific target genes and regulate transcription. However, studies have revealed that STAT3 can pass across the nuclear membrane even in an unphosphorylated state and that it dynamically shuttles between cytoplasmic and nuclear compartments, acting in the nucleus as a transcriptional activator/repressor and a chromatin/genomic organizer (Timofeeva et al., 2012; Yang et al., 2005). By analogy, however, masking of the dimerization site by binding of importin-α5 to unphosphorylated STAT1, suggests that nuclear importation through this mechanism would only function

for unphosphorylated STAT3 monomers (Reich and Liu, 2006). Moreover, STAT3 has been found to contain a constitutive nuclear localization signal (NLS) within its coiled-coiled domain which can be recognized by the importin-α3 and -α6 nuclear transport factors regardless of its phosphorylation status (Liu et al., 2005). Once in the nucleus, STAT3 can be phosphorylated on serine residue 727 (Ser-727) by several kinases, including ERK1, ERK2, p38, JNK, and protein kinase C (PKC) (Decker and Kovarik, 2000). This phosphorylation is thought to increase the DNA binding affinity of STAT3 (Ng and Cantrell, 1997; Zhang et al., 1995). The requirement for a phosphorylated Ser-727 in STAT3 may vary depending on the the target promoter subject to transcriptional regulation and/or cellular types, and phosphorylation of this residue can occur independently to that of the Tyr-705 residue. Molecules of activated STAT3 then interact with transcriptional co-activators, such as histone acetyltransferases, to induce transcription. Alternatively, they can associate with protein complexes containing histone deacetylases to induce transcriptional repression.

The gene set regulated by STAT3 is thought to vary between cell types and differentiation stage of the cell in question. A recent study by Diego Miranda-Saavedra's group clearly illustrated this concept. They studied STAT3 genome-wide binding patterns in four different cell types: embryonic stem cells, $CD4^+$ T cells, macrophages and AtT-20 corticotroph cells. They found that only a very small number of STAT3-binding sites were common to all four of the cell types analyzed. Binding to these sites enabled regulation of a core set of genes

that are pre-bound by MYC, E2F1 and other transcriptional factors. In addition, they showed that most of the genes bound by STAT3 are cell type-specific and therefore responsible for the distinct biological outcomes of STAT3 activation in the various different cell types (Hutchins et al., 2013). These differences may be explained by the binding of STAT3 to variant DNA motifs or by the different transcriptional regulatory modules able to assemble around STAT3 in the different cell types to drive distinct transcriptional programs, as has been demonstrated for other transcription factors, such as SMAD2/SMAD3 (Mullen et al., 2011). This would mean that the interactions between STAT3 and associated transcription factors, as a result of either direct protein-protein interactions or resulting from binding of neighbouring sequences, may be responsible for the distinct patterns of gene induction in different cell types. In order to examine the consequences of STAT3 activation in human TEL-AML1^{+} cells, we decided to examine changes in the expression of potential candidate target genes upon STAT3 inhibition mediated by S3I-201 treatment or shRNA silencing.

6.1.1 SURVIVIN and MYC are transcriptionally regulated by STAT3

Although STAT3 controls distinct subsets of genes in different cancer cells, a few of the target genes are common to most. Among these there are genes encoding anti-apoptotic proteins, such as SURVIVIN and members of the Bcl-2 family (e.g., Bcl-XL, Bcl-2), and proteins involved in proliferation and

cell cycle progression, such as cyclin D1 and c-MYC (Bromberg and Darnell, 2000; Dauer et al., 2005). In order to test the downstream effect of STAT3 inhibition in TEL-AML1 leukaemia we decided to examine the expression of *SURVIVIN* and *MYC*, two well-characterized STAT3 target genes that have been described to play important role in both normal haematopoiesis and in cancer.

SURVIVIN, also known as BIRC5, is a member of the inhibitor of apoptosis (IAP) protein family. The main function of this protein is to inhibit cell death induced by caspase activation, however the mechanisms by which SURVIVIN protects from apoptosis still remain unclear (Li et al., 2008). In addition, it also functions in regulating cell division due to its ability to bind microtubules of the mitotic spindle through its carboxyl-terminal alpha helices (Li et al., 1999). *SURVIVIN* expression is limited to embryonic tissues, to cells undergoing differentiation and to most cancer cells, whereas in terminally differentiated cells its expression is almost completely absent (Ambrosini et al., 1997). During the cell cycle, SURVIVIN is expressed in the G2/M phase and promotes the proper segregation of chromosomes (Lens et al., 2003). Due to its function in cell division and apoptosis, *SURVIVIN* is overexpressed in many cancers and its expression has been correlated with drug-resistance in particular tumours (Zaffaroni and Daidone, 2002). In t(12;21) leukaemia, a direct link has been established between the TEL-AML1 fusion protein and *SURVIVIN*. Thus, TEL-AML1 silencing was shown to result in reduced *SURVIVIN* expression (Diakos et al., 2007; Diakos et al., 2010), its regulation

being mediated via two different miRNAs (miRNA-494 and miRNA-320a) (Diakos et al., 2007; Diakos et al., 2010). *SURVIVIN* has been shown also to be a direct transcriptional target of STAT3 in different models (Aoki et al., 2003; Gritsko et al., 2006; Sen et al., 2012).

c-MYC was discovered in 1982 as the gene encoding a transcription factor homologue of the viral oncogene (*v-MYC*), from the avian myelocytomatosis retrovirus (Vennstrom et al., 1982). *c-MYC* belongs to the *MYC* family of transcription factor genes, which also include the *n-MYC* and *l-MYC* genes. As in STAT3 signalling, many different mitogenic factors and growth promoting stimuli converge upon c-MYC, stimulating and regulating both its expression and transcription factor activity. For this reason, it plays a fundamental role in replication, growth, apoptosis and differentiation. Targeted homozygous deletion of the murine *c-Myc* gene resulted in embryonic lethality, suggesting a critical function during development (Davis et al., 1993). Interestingly, foetal death occurred around day 10.5, coincident with the onset of foetal liver haematopoiesis, suggesting an important function of c-MYC in regulating correct haematopoietic development. Significantly, one of the main characteristics in these mice was the presence of circulatory defects. A study of mouse embryos conditionally deficient in *c-Myc* expression, specifically in the haematopoietic lineage, showed the same phenotype as those lacking *c-Myc* in the entire embryo, confirming its role in this developmental process. Thus, deletion of *c-Myc* solely in haematopoietic cells, resulted in embryonic lethality due to a failure in normal development of haematopoietic cells, that

resulted in a defect in angiogenesis (He et al., 2008). It has been shown, moreover, that not all MYC family members are expressed in haematopoietic stem/progenitor stem cells and those members that are expressed are always co-expressed with *c-MYC* (Laurenti et al., 2008). In adult haematopoiesis, *c-MYC* plays an important role for stem cell maintenance and differentiation. It controls the interaction between HSCs and the bone marrow niche by regulating expression of many adhesion-related molecules, including N-cadherin and integrin-β1, -α2, and -α5, in HSCs (Wilson et al., 2004). *c-MYC* also has a role during the differentiation of both lymphoid and myeloid cells. It is expressed, together with *n-MYC*, during the maturation and expansion of the earliest B precursor cells, and constitutive expression of c-*MYC* has been shown to result in elevated numbers of B progenitors. On the other hand, compound deletion of c-*Myc* and *N-Myc* family members caused a block in B cell development at the pro-B to pre-B transition (Habib et al., 2007). This suggests a possible link between MYC function and STAT3 signalling in regulating B cell maintenance and expansion, since conditional deletion of *Stat3* in progenitor cells in the bone marrow results in a block at the same stage of B cell development (Delgado and Leon, 2010). A link between c-MYC and STAT3 has been well established. Firstly, different studies have shown that *c-MYC* is a direct transcriptional target of STAT3 (Bowman et al., 2001; Cartwright et al., 2005; Kiuchi et al., 1999; Ling and Arlinghaus, 2005). Interestingly, experiments using a murine pro-B cell line (Baf-3) showed that activation of STAT3 leads to increased c-MYC expression (Kiuchi et al., 1999).

Secondly, it can function as cooperating factor to regulate STAT3 transcriptional activity by binding at the same time DNA sequences in the same promoter synergizing or modulating the gene expression (Barre et al., 2005; Hutchins et al., 2013).

Constitutive or deregulated expression of c-MYC is a characteristic of most cancers. For example, genetic alterations in *c-MYC* have been reported to contribute to one-seventh of U.S. cancer deaths (Dang, 1999). The first human tumour in which c-MYC deregulation was identified was Burkitt's lymphoma (Dalla-Favera et al., 1982; Taub et al., 1982). *c-MYC* has since been shown to be altered or its expression up-regulated in many others haematological diseases, including both adult and childhood ALL (Delgado and Leon, 2010; Faderl et al., 2010). Taken together, these reports suggest that c-MYC may be critically important in malignant as well as normal haematopoiesis.

6.2 Results

6.2.1 c-MYC is transcriptionally regulated by STAT3 in TEL-AML1 leukaemia

In addition to its reported function in malignant haematopoiesis, *c-MYC* is also a well characterised transcriptional target of STAT3 signalling. For these reasons, we decided to examine whether *c-MYC* expression was regulated by STAT3 signalling in t(12;21) ALL. We treated TEL-AML1^{+} cell lines and the

non-TEL-AML1$^+$ cell line 697, with S3I-201 for 6 hours in order to isolate mRNA, and 24 hours for protein analysis. This analysis demonstrated a marked reduction in *c-MYC* mRNA (Figure 6-1A) and complete loss of c-MYC protein in Reh and At-2 cells following exposure to 50μM S3I-201 (Figure 6-1B). In contrast, there was only a modest reduction in *c-MYC* mRNA (Figure 6-1A) and protein in 697 cells (Figure 6-1C). According to our model, activation of STAT3 in TEL-AML1 leukaemia is mainly mediated by RAC1. In order to further validate this hypothesis we decided to test the expression of *c-MYC* upon treatment with NSC 23766. The results showed a reduction of both mRNA and protein similar to that obtained by inhibiting STAT3 (Figure 6-2A and 2B), suggesting a strong dependence of *c-MYC* expression upon RAC1 activity. Importantly, the reduction in c-MYC expression in 697 cells was detectable only with S3I-201 treatment, but not with NSC 23766 (Figure 6-1A, 2A), indicating that this signalling pathway may be specific to TEL-AML1 leukaemia.

Since S3I-201 treatment of TEL-AML1$^+$ cells results in apoptosis, it is possible that the reduction in MYC protein expression observed was an indirect result of the loss in cell viability. In order to examine this possibility, we measured MYC protein expression in Reh cells after 4 hours exposure to S3I-201, at which point there was no detectable induction of apoptosis (Figure 6-3A). Despite the lack of apoptotic response at this time-point, S3I-201 treatment nevertheless caused a significant reduction in MYC protein expression (Figure 6-3B). This suggests that the reduction in MYC expression

is due to a direct effect of STAT3 inhibition, rather than non-specific cell death. Down-regulation of c-MYC protein was also confirmed after shRNA-mediated *STAT3* silencing (Figure 6-4A). Furthermore, silencing of *TEL-AML1* also resulted in reduced c-MYC expression (Figure 6-4B), confirming the function of this fusion protein in maintaining elevated STAT3 activity in leukaemic cells.

Although these data indicate that c-MYC expression requires STAT3 signalling in TEL-AML1$^+$ cells, they do not address the importance of c-MYC expression for growth and survival of these cells. To examine the importance of c-MYC expression in TEL-AML1 leukaemia, we decided to analyse the effect of silencing *c-MYC* directly in TEL-AML1$^+$ cell lines cells (Figure 6-5A). Knock-down of *c-MYC* in Reh cells resulted in similar induction of apoptosis and inhibition of cell cycle progression as that observed after *STAT3* knock-down (Figure 6-5B, C). Furthermore, it also resulted in a complete a loss of self-renewal activity in colony forming assays (Figure 6-6). This suggested that *c-MYC* silencing may impair the ability of Reh cells to engraft leukaemia *in vivo*. In order to examine this possibility, Reh cells were transduced with control scramble or *c-MYC* shRNA expressing vectors and 48 hours later an equivalent number of viable cells were transplanted into sub-lethally irradiated recipient NSG mice. As predicted, *c-MYC* silencing resulted in a prolonged latency of leukaemia development *in vivo* (Figure 6-7). Despite these data, it was nevertheless possible that the requirement for STAT3 induced *c-MYC* expression in the TEL-AML1$^+$ cell lines was associated with their proliferative status. For this reason we also examined c-MYC expression in two primary

patient t(12;21) ALL samples following exposure to S3I-201. Although these leukaemia cells samples did not proliferate when placed *in vitro*, S3I-201 treatment abolished c-MYC protein expression, consistent with the data obtained in Reh cells (Figure 6-8).

6.2.2 SURVIVIN is transcriptionally regulated by STAT3 in TEL-AML1 leukaemia

In order to examine the effect of STAT3 inhibition on *SURVIVIN* expression, we measured the level of *SURVIVIN* mRNA expression in TEL-AML1$^+$ cell lines after 6 hours exposure to 50μM S3I-201, and protein levels 24 hours after exposure. In both cases, inhibition of STAT3 activity resulted in reduced levels of SURVIVIN expression in the cells (Figure 6-9A, B). Moreover, in order to show the validity of the RAC1-STAT3 pathway in downstream regulation of *SURVIVIN* expression, we analysed gene expression after RAC1 inhibitor treatment. Treatment of cells with NSC 23766 resulted in similar reductions of *SURVIVIN* expression, confirming upstream regulation of STAT3 activity via RAC1 (Figure 6-9C).

6.3 Discussion

Although the target genes transcriptionally regulated by STAT3 may vary between different cell types and cancers, our data demonstrate that in TEL-AML1$^+$ ALL there is an absolute requirement for STAT3 activity in the maintenance of expression of two established target genes: *BIRC5* and *c-MYC*. We focussed our experiments on *c-MYC*, given the widely reported oncogenic activity this gene. Our results demonstrate that loss of *c-MYC* expression in TEL-AML1$^+$ cells causes cell death and, importantly, a significant delay of leukaemia development *in vivo*.

Aberrant expression of c-MYC has been demonstrated previously in paediatric acute lymphoblastic leukaemia, including t(12;21) ALL, by Malempati et al (Malempati et al., 2006). In this study, analysis of protein stability revealed that the half-life of c-MYC protein was prolonged in the majority of the cases. Contrary to what has been observed in other diseases, such as in Burkitt's lymphoma (Bahram et al., 2000), this increase in stability was not due to the presence of somatic mutations in the c-MYC gene, but was caused by deregulation of pathways controlling the phosphorylation of threonine 58 and serine 62, the two amino acid residues that determine c-MYC stability (Malempati et al., 2006). Our data that suggest that increased c-MYC expression may also result from transcriptional induction of *c-MYC* gene expression by STAT3 activation. Taken together, these data indicate a fundamental role for c-MYC in TEL-AML1 leukaemia.

A new concept for how c-MYC functions in transcriptional regulation have recently emerged. Two papers showed that c-MYC acts as a non-linear amplifier of gene expression rather than functioning as a traditional transcription factor (Lin et al., 2012; Nie et al., 2012). Using genome-wide analysis of c-MYC gene occupancy and the resulting changes in gene expression, the authors of both studies concluded that c-MYC associates with promoters of genes that are already actively transcribed and that this association enhances transcriptional output further (Lin et al., 2012; Nie et al., 2012). If this concept is true, the requirement for elevated c-MYC expression in TEL-AML1 leukaemia could be explained by the possibility that it is essential for the transcriptional activity of TEL-AML1 itself, thereby enhancing the expression of genes regulated by the fusion protein.

Requirement of c-MYC up-regulation or stabilization could be also necessary for the increased metabolic activity of the leukaemic cells. One of the main characteristics of all cancer cells is the increased rate of proliferation in comparison to normal cells. In order to increase the rate of cell division the cells need to alter their metabolic activity to provide the necessary energy. This metabolic shift has been well studied in solid tumour cells but there is evidence that it also occurs in childhood pre-B acute lymphoblastic leukaemia. Thus, Boag and colleagues demonstrated an altered expression of genes involved in glycolysis and tricarboxylic acid (TCA) cycle in cells from ALL patients compared to normal $CD34^+$ cells (Boag et al., 2006). For example, there was a significant up-regulation in expression of genes coding for glucose transport

proteins (GLUT). These findings suggest that ALL cells may have a similar metabolic profile to solid tumour cells. c-MYC plays an import role in this process and has been shown to directly target many genes involved in glycolysis, including *GLUT1* (Dang et al., 2009). In addition to its role in regulation of genes involved in glycolysis, c-MYC appears to increase mitochondrial function, necessary for cell division. It has been shown, for example, that c-MYC increases mitochondrial synthesis of acetyl-CoA which, in turn, contributes to significant increases in histone acetylation and fatty acid biosynthesis that allow the cells to divide faster (Morrish et al., 2010).

In conclusion c-MYC expression is essential for the survival of TEL-AML1 leukaemia. However, further experiments are needed to clarify its role in the regulation of gene expression changes induced by the fusion protein and also to understand how it functions to modify metabolic activity in the transformed cells.

6.4 Figures

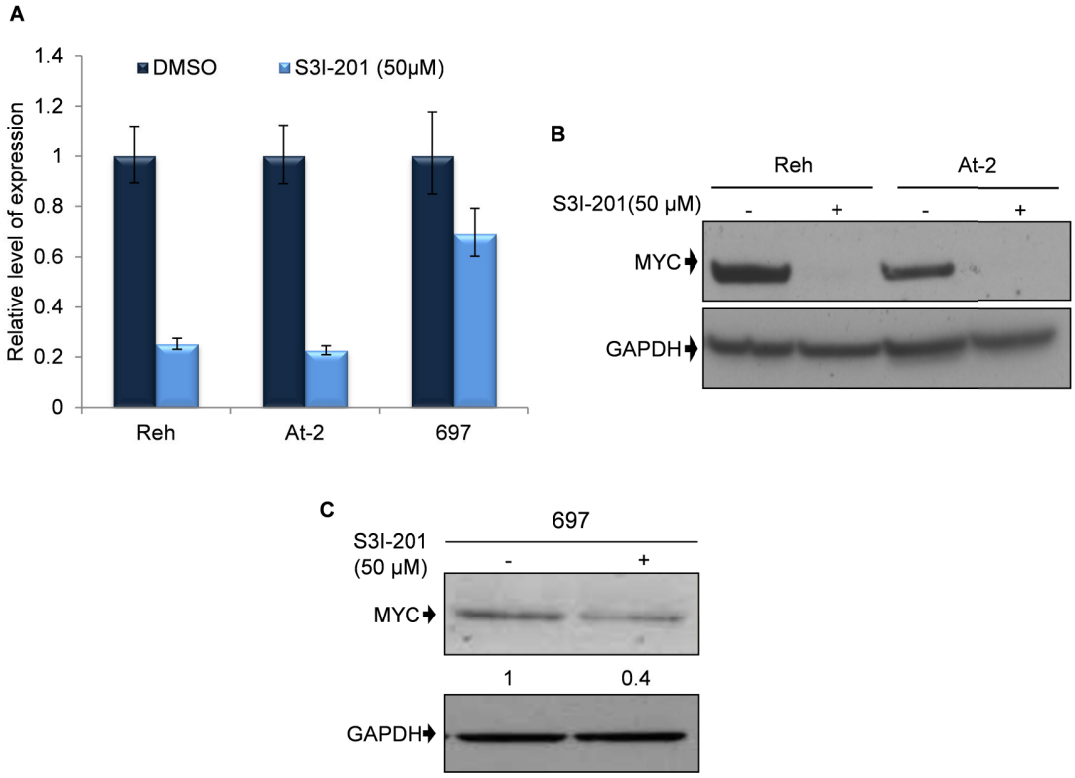


Figure 6-1: S3I-201 treatment causes reduced levels of MYC mRNA and protein in TEL-AML1^{+} but not in TEL-AML1^{-} cell lines.

The figure shows the reduction of *MYC* mRNA (**A**) and MYC protein (**B** and **C**) in TEL-AML1^{+} but not in TEL-AML1^{-} cell lines following 50μM S3I-201 treatment. The cells were cultured in presence of the STAT3 inhibitor for 6 or 24 hours prior to mRNA and protein analysis, respectively. Numbers represent densitometric quantitation of MYC bands normalized to GAPDH. All data are representative of three independent experiments and show means ± s.d. of quadruplicate measurements.

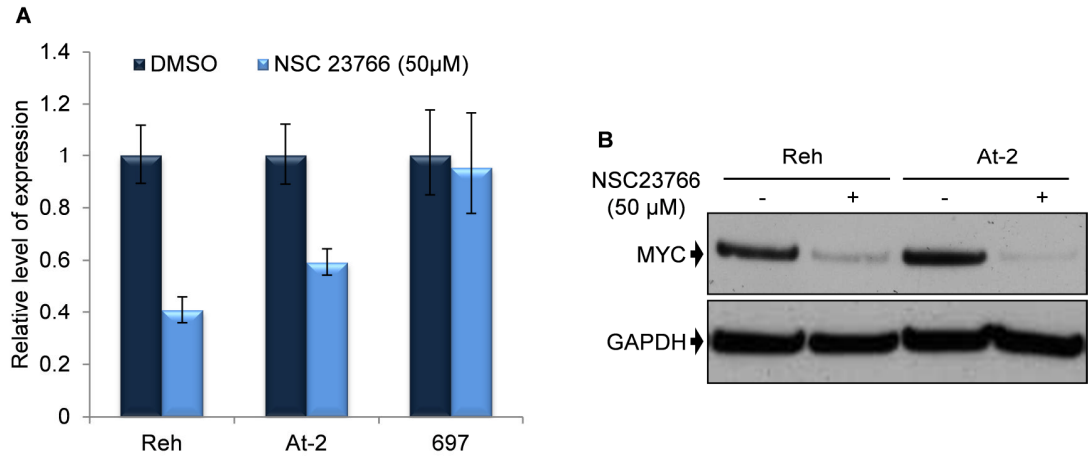


Figure 6-2: NSC 23766 causes reduced levels of MYC mRNA and protein in TEL-AML1^{+} but not in TEL-AML1^{-} cell lines.

The figure shows the reduction of *MYC* mRNA (**A**) and MYC protein (**B**) in TEL-AML1^{+} but not in TEL-AML1^{-} cell lines following 50µM NSC 23766 treatment. The cells were cultured in presence of the RAC1 inhibitor for 6 or 24 hours prior to mRNA and protein analysis, respectively. All data are representative of three independent experiments and show means ± s.d. of quadruplicate measurements.

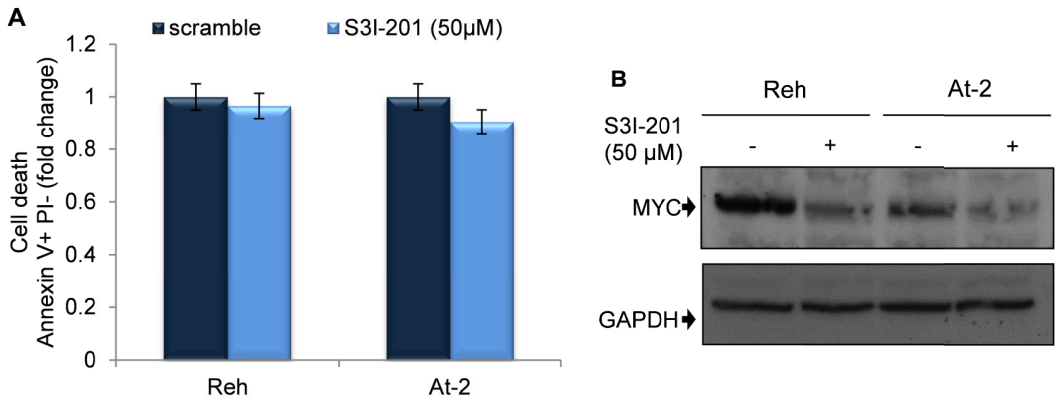


Figure 6-3: Reduction of MYC protein is not a consequence of apoptosis.

The figure show the analysis of MYC protein after 6 hours of 50M S3I-201, at which time no apoptosis can be detected (**A**). The western blot analysis displays a significant reduction of MYC protein (**B**). All data are representative of three independent experiments and show means ± s.d. of triplicate measurements.

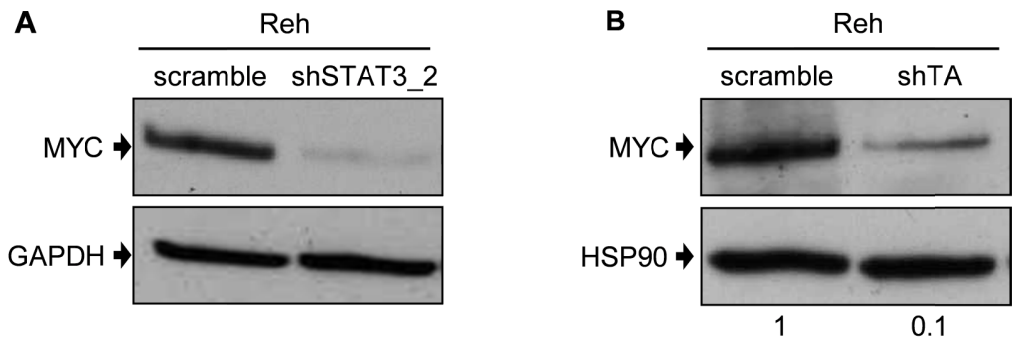


Figure 6-4: STAT3 and TEL-AML1 regulate the expression of MYC.

Western blot analysis of MYC protein expression after lentiviral transduction with scramble control and shSTAT3 (**A**) or shTA (**B**) shRNA. Numbers represent densitometric quantitation of MYC bands normalized to GAPDH (**A**) or HSP90 (**B**). All data are representative of three independent (**A**) or two independent (**B**) experiments.

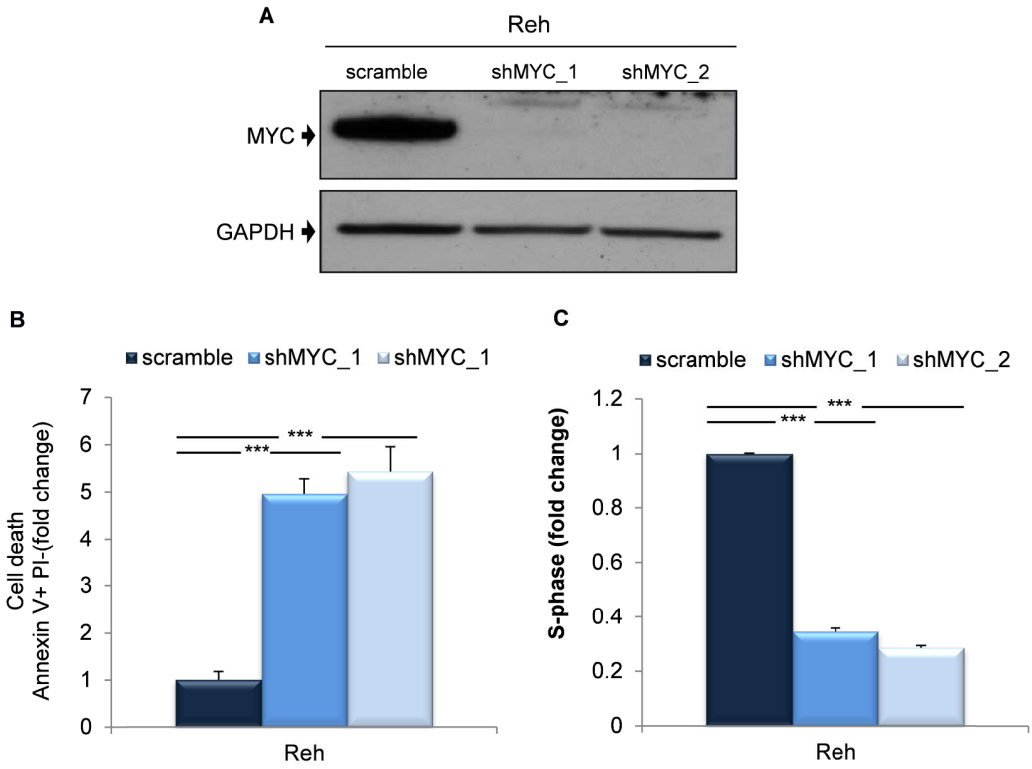


Figure 6-5: shRNA mediated MYC silencing in Reh results in apoptosis and cell cycle block.

The figure show Western blot analysis of MYC protein expression in Reh cells (**A**) 5 days after transduction with control scramble or two different *MYC* shRNAs. The bar charts represent percentage of apoptotic Annexin V^+PI^- (**B**) and percentage of cells in the S-phase of the cell-cycle (**C**) six days after transduction with control scramble or *MYC* shRNAs in Reh. Cells were pulsed with 10μM 5-ethynyl-2´-deoxyuridine (Edu) for 1 hour. All data are representative of three independent experiments and show means ± s.d. of triplicate measurements. **P* < 0.05, ***P* < 0.01, ****P* < 0.005 compared to control (Student's unpaired *t* test).

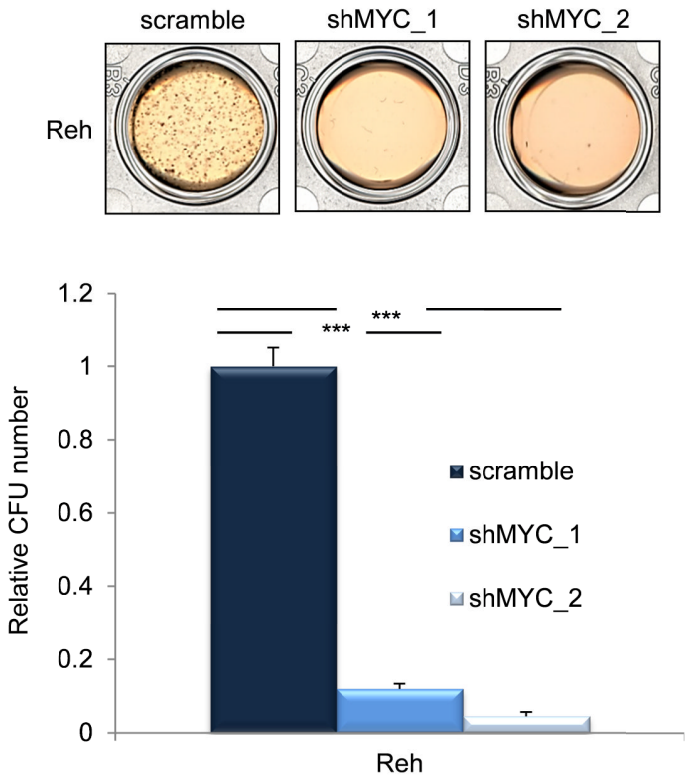


Figure 6-6: *MYC* silencing impairs the self-renewal ability of Reh cells.

The figure shows Reh CFU (upper panel) following *MYC* silencing and quantification of the number of colonies formed (lower panel). The cells were plated at $0.18x10^4$ cells per well in a 24 multiwell plate, 5 days after lentiviral transduction and stained with INT after 14 days. All data are representative of three independent experiments and show means ± s.d. of triplicate measurements. *P < 0.05, **P < 0.01, ***P < 0.005 compared to control (Student's unpaired t test).

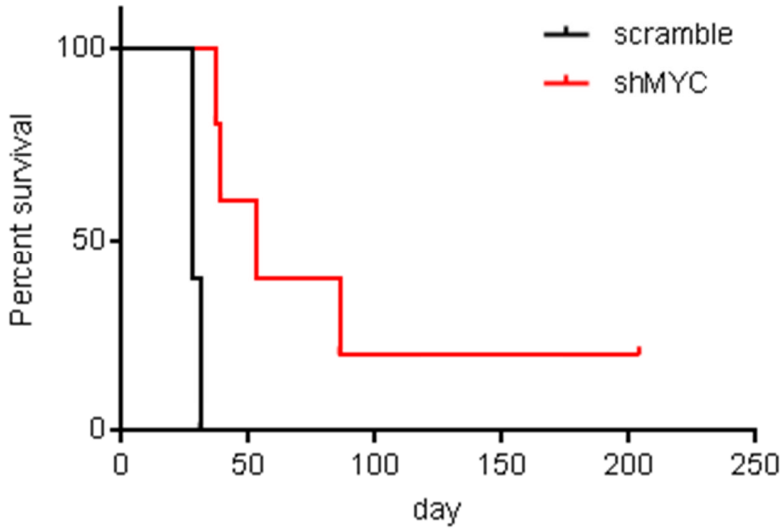


Figure 6-7: *MYC* silencing impairs engraftment of Reh cells *in vivo*.

The figure shows the Kaplan-Meier survival curve for NSG mice transplanted with control scramble (n=5) or shMYC (n=5) transduced Reh cells.

p= 0.002 versus scramble control (Mantel-Cox log rank test).

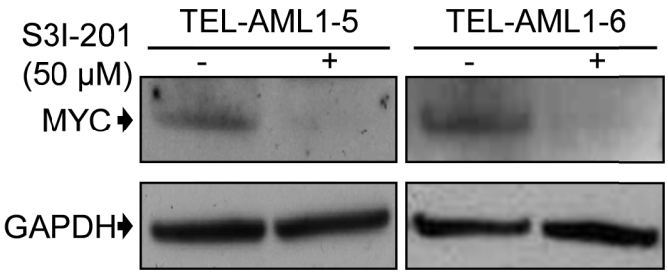


Figure 6-8: Pharmacological inhibition of STAT3 causes MYC protein loss in primary TEL-AML1$^+$ samples.

The figure shows the loss of MYC protein in two different primary TEL-AML1$^+$ samples after 24 hours of 50μM S31-201 treatment.

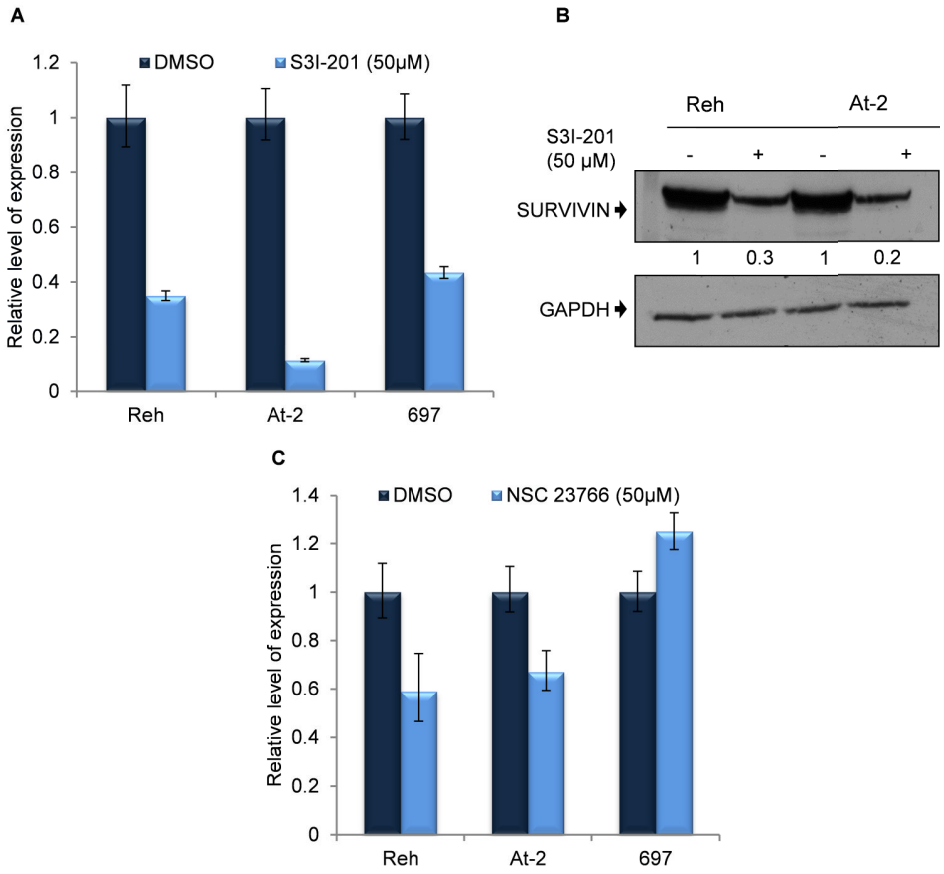


Figure 6-9: STAT3 and RAC1 inhibition causes reduced levels of SURVIVIN mRNA and protein in TEL-AML1$^+$ but not in TEL-AML1$^-$ cell lines.

The figure shows the reduction of *SURVIVIN* mRNA (**A**) and protein (**B**) in TEL-AML1$^+$ but not in TEL-AML1$^-$ cell lines following 50μM S3I-201 treatment. The same reduction is observed after NSC 23766 treatment (**C**). The cells were cultured in presence of the inhibitors for 6 or 24 hours prior mRNA and protein analysis, respectively. Numbers represent densitometric quantitation of SURVIVIN bands normalized to GAPDH. All data are representative of three independent experiments and show means ± s.d. of quadruplicate measurements.

CHAPTER VII

7.1 Conclusions

The TEL-AML1 fusion gene has been considered to be a weak oncogene and insufficient on its own for leukaemic transformation. Different studies, including some carried out by our group, have previously demonstrated that its presence is able to perturb haematopoietic pathways promoting *in vitro* B cell differentiation and predispose haematopoietic stem cells to generate overt leukaemia after acquisition of secondary genetic alterations (Morrow et al., 2004; Schindler et al., 2009). The identification of the specific pathways altered by the fusion gene itself has only recently become a focus of study. The data collected so far, however, have only partially demonstrated an active role for TEL-AML1 in deregulating specific pathways. In 2009, for example, Ford and colleagues demonstrated that TEL-AML1 is able to reduce the sensitivity of haematopoietic cells to TGFβ (Ford et al., 2009). Analysis of gene expression after *TEL-AML1* silencing in human TEL-AML1^{+} leukaemic cell lines revealed an active function of the fusion protein in activating the PI3K/AKT/mTOR pathway. However, this study did not describe how this is achieved (Fuka et al., 2012).

In this study, we demonstrate a novel signalling pathway important for the maintenance of TEL-AML1 leukaemia and, for the first time, an active role of TEL-AML1 in directly regulating an oncogenic pathway (Fgure 7.1). In our model, TEL-AML1 activates RAC1 which, in turn, induces STAT3 activation.

STAT3 activation is necessary for the survival, proliferation and self-renewal of TEL-AML1 positive leukaemia, through transcriptional induction of *MYC* expression. We demonstrate, in fact, that human leukaemic cell lines carrying this translocation are highly sensitive to treatment with S3I-201, a specific STAT3 inhibitor, and that primary human leukaemic samples are also responsive to this drug.

Although clinically, TEL-AML1 positive patients have excellent outcomes, relapses are reported to occur in 10-20% of children and at the moment there is insufficient data on the long-term effects of chemotherapy. STAT3 inhibition may hence provide an alternative therapeutic approach capable of overcoming both of these problems. STAT3 targeted therapy in TEL-AML1 leukaemia may in fact have several beneficial effects. First, short-term STAT3 treatment has been shown to not have severe side effects (Kortylewski et al., 2005; Lee et al., 2011). Second, treatment with STAT3 specific inhibitors has been associated with an increment of innate and adaptive immune responses to cancers (Kortylewski et al., 2005; Lee et al., 2011). This may be as important as the direct inhibitory effects of the drug on the leukaemic cells since there is increasing evidences for how the microenvironment could influence leukaemic transformation and maintenance of cancer clones (Purizaca et al., 2012). Third, although MYC is one of the most de-regulated proteins in human cancers, targeting this protein has been always a challenge. Its ubiquitous expression in all proliferating cells, suggesting that its inhibition may be associated with unacceptable toxicities,

and the difficulty in designing efficacious inhibitors have been the main reasons for the failure of MYC targeting (Prochownik and Vogt, 2010). STAT3 inhibition could be a way to bypass these difficulties in targeting MYC in TEL-AML1 leukaemia.

Our data propose that activation of RAC1 is mainly mediated by the specific GEF ARHGEF4. Inhibition of the *ARHGEF4* gene by shRNA mediated silencing results in complete cell death and loss of engraftment in NSG mice, suggesting that ARHGEF4 targeting could be of therapeutic interest. However, with the current technology available, the shape, structural dynamics and chemistry of GEF–GTPase interaction surfaces make it very difficult to develop small molecule inhibitors (Vigil et al., 2010). Our results suggest that regulation of ARGHEF4 in TEL-AML1 leukaemia is mediated by transcriptional activity of the fusion gene. However, transcriptional regulation leading to elevated ARHGEF4 expression alone may not be sufficient to fully activate the RAC1 protein. Moreover, in addition to being a specific activator of RAC1, ARHGEF4 may have an independent function in regulating the survival and proliferation of leukaemic cells. For this reason developing new tools to study the function of TEL-AML1 in regulating ARHGEF4 and the latter's role in this subtype of ALL will be promising in order to develop new therapeutic strategies to improve the treatment of TEL-AML1 leukaemia.

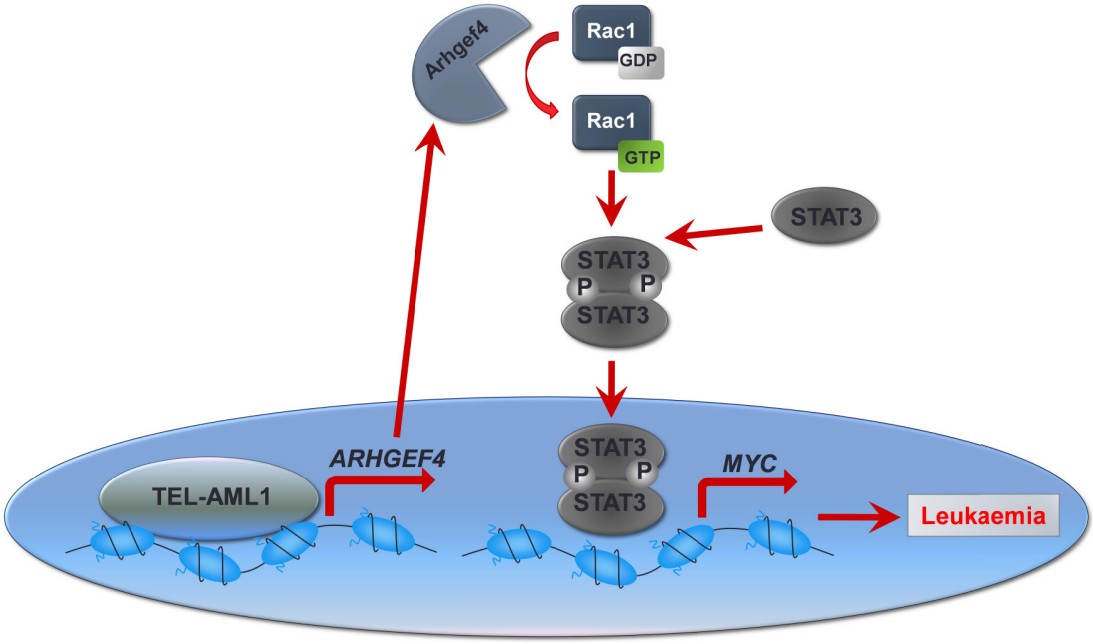


Figure 7-1: Novel oncogenic signalling pathway in TEL-AML1 leukaemia. In our model TEL-AML1 is able to regulate the activation of RAC1 through transcriptional regulation of *ARHGEF4*. This activation is sufficient to increase the phosphorylation levels of the signal transducer and activator of transcription 3 (STAT3) and consequent regulation of *MYC* expression. The presence of activated STAT3 is necessary for the survival, proliferation and self-renewal of TEL-AML1 leukaemia allowing the leukaemic clone to survive.

CHAPTER VIII

8.1 References

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APPENDIX